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BIOLOGICAL BULLETIN

MODIFICATION OF DEVELOPMENT ON THE BASIS OF DIFFERENTIAL SUSCEPTIBILITY TO RADIATION.

II. Arbacia and Visible Light following Sensitization.

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Previous experiments by Bovie and Barr, '24, and by Hinrichs, '24, have clearly demonstrated that living organisms are differentially susceptible to lethal doses of radiation as well as to lethal doses of other physical and chemical agents (Child, '24). Those regions of the body which are physiologically the most active, are the first to die and disintegrate. There is a progressive antero-posterior disintegration gradient, coincident in lower animals and embryonic stages, with the main body axis. Disintegration gradients, as demonstrated by the method of photolysis, are essentially like those produced by other chemical and other physical agents.

With sublethal doses of radiation, as with sublethal doses of other agents, it is possible to modify embryonic development (Child, '24*b*, and Hinrichs, '24, and '25, now in press). With the proper intensity and duration of exposure, the developmental rates of various parts of the embryo may be relatively altered. Here again the development of those parts which are physiologically the most active, is most easily inhibited or entirely suppressed when doses are used which produce a permanent inhibition, and do not permit recovery or acclimation. On the other hand, if the inhibitory effect is slight or transitory, these same regions of high physiological activity will be the first to acclimate or recover. (See Child, '24*b*, p. 247, for review of literature.)

Differential inhibition and differential recovery have been brought about (1) through the use of visible radiation (diffuse daylight, direct sunlight filtered through a glass dish containing water an inch deep, artificial light from two 1,500-watt photographic bulbs, and from the white-flame carbon arc) *plus* the action of the following sensitizing dyes: eosin, neutral red, benzoflavine, and methylene blue; and (2) by means of radiation from a mercury-vapor arc. (See also Child, '24*b*, footnote, p. 109.) Differential acclimation has been brought about by continuous exposure of sensitized eggs to diffuse daylight.

The development of eggs of Echinoderms has been repeatedly modified experimentally,[1] but the fact that there are relative differences in degree of susceptibility to modification of the various regions along the embryonic axes has not been generally noted. (For a more complete discussion of the results obtained by differentially modifying development, see Child, '16 and '23.) The main conclusions reached by this author regarding the forms attained in this way may be summed up as follows:

1. "In cases of differential inhibition the chief changes are decrease in size of oral lobe, which represents the apical region, decrease to zero in the angle of divergence between arms, approach of lateral parts toward the median line and in more extreme degrees fusion in the median line, the progressive obliteration of antero-posterior and medio-lateral differences and finally of apico-basal differences.

2. "The changes in differential acclimation and differential recovery are in the opposite direction. They consist in increase in size and over-development of the oral lobe, increase to 180° in the angle of divergence between the arms, and over-development of anterior and median as compared with posterior and lateral regions.

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[1] For literature on modification of development see the following;

(*A*) Visible radiation following sensitization—Cooke and Loeb, '08, '09, Shippen, '07, and Loeb, '07, *a*, *b*.

(*B*) Ultraviolet radiation—Baldwin, '15, Stevens, '09, Ruppert, '24, Child, '24*b*, footnote, p. 109, Clarke, '22.

(*C*) X-rays and radium—Loeb, '22, Colwell and Russ, '24, Hinrichs, '25 (unpublished).

3. "Where the effect of differential inhibition persists after general recovery, a great over-development of the skeleton may occur, since the mesenchyme cells are relatively less inhibited than most, if not all other parts."

Larvæ whose development has been modified by means of radiation, present exactly the same picture as those produced by other means.

Method.—In general, in experiments in which differential inhibition has been produced, the method has been as follows: The eggs from a single female were strained through a cheese-cloth and washed. They were then stained in weak sea-water solutions of one or the other of the above dyes, for from ten to thirty minutes, again washed and then exposed for varying periods of time to radiation from one of the above-mentioned sources of light. (In some cases the eggs were exposed in a thin layer of a weak concentration of the dye.) Eggs were then washed and returned to sea-water and allowed to develop in the dark. The experimental controls consisted of (*A*) stained and unstained eggs (unexposed to strong light) which were allowed to develop in diffuse daylight, and another similar lot which developed in the dark, and (*B*) unstained eggs (exposed to strong light) and allowed to develop in the dark. Temperature was controlled by the addition of ice to the water bath surrounding the eggs.

Differential recovery followed in those cases where the inhibition was only transitory. Differential acclimation was obtained by allowing fertilized eggs to develop in sea-water solutions of dyes exposed to diffuse daylight.

Results.—Results of experiments made during the summers of 1923 and 1924 may be tabulated as follows:

A.	Differential inhibition	no recovery.
B.	Differential inhibition	differential recovery.
C.	Differential inhibition	general recovery.
D.	Differential acclimation.	

A. *Differential Inhibition*.—Since it is true that those regions which are relatively the most active physiologically, during a particular stage of growth, are also the most highly susceptible to the destructive action of lethal doses of chemical agents

(Child, '16, '20, '23), and since these regions are also the first to be modified by sublethal doses of such agents (Child, '16*b*, '24), it seemed likely that those regions of the developing embryo which showed the greatest degree of developmental modification (see also Child, '16*b*), as for example, the oral lobe and aboral arms, would also show the earliest evidences of disintegration in lethal doses of visible light following sensitization (Figs. 1–3).

Modification of development in these regions is brought about by an exposure made much earlier than at the pluteus stage. A short exposure made immediately after fertilization will produce abnormal inhibited development of these active regions. Two possibilities suggest themselves; either a general metabolic lowering of developmental rate at the time of exposure, produces an effect at first latent, and not appearing until later, at a highly susceptible stage of development; or, there is an early difference in the relative susceptibility of regions or "Anlagen" present in the fertilized egg. The latter seems more probable. (See also Hinrichs, '25, now in press.)

As a result of inhibition of development by means of radiation, forms are produced in which the anterior region of the developing embryo is relatively less developed than other regions. The oral lobe, which represents the apical region, and the aboral arms which together with the oral lobe, forms the anterior region of the pluteus, show the greatest effect (Figs. 7–11). The angle between the arms is greatly reduced. Often there is but a single median arm (Figs. 12, 13) or one partially fused anteriorly (Fig. 14).

If the inhibitory effect has been even more marked, the aboral arms and oral lobe may be undeveloped, and there remains an ovoid mass which may have short parallel skeletal arms embedded in the tissue, one on either side of the archenteron, which latter has but one opening to the exterior (Fig. 15). A final stage of inhibition shows an apolar spherical mass with non-directive swimming (Fig. 16). Exogastrulæ (Figs. 17–22) are also commonly found in cultures in which inhibition has been produced. (See also Herbst, '95, and MacArthur, '24.)

B. Differential Recovery.—As examples of differential recovery obtained by short early exposures in which the harmful effect is transitory, wide-angled forms, some up to 180°, with over-developed oral lobes give evidence that the median anterior region has recovered from a slight inhibition and that its rate of growth has surpassed that of other regions (Figs. 23–27).

C. General Recovery.—Individual differences in eggs from the same female (see also Medes, '17, and Tennent, '10) produce differences in degree, and even in direction of effect; but the larger number (90 per cent. or more) of the embryos in a given lot may, for example, show differential inhibition, while the rest of the embryos in the lot may show general recovery or no effect at all. The degree of effect produced is roughly proportional to the dosage (see Figs. 58, 63) and is also greater at higher temperatures. (See Bovie and Daland, '23, Bovie, '18.)

D. Differential Acclimation.—Differential acclimation results when the inhibitory effect of radiation is mild enough to permit adjustment, for example, when the eggs are exposed continuously, in weak concentrations of dyes, to diffuse daylight, or are kept in stronger concentrations for several days in the dark. The resulting forms, are wide-angled and have over-developed oral lobes like those appearing after differential recovery. (See Figs. 37–39.)

In higher concentrations there is a differential effect on development even in the dark (Figs. 33–39); but in order that the lower concentrations may be effective, there must have been at some time, preferably during early development, an exposure of sufficient duration to radiation of the proper intensity. The effects produced under these conditions can to some extent be controlled.

Frequently there appears a larger number of aboral arms than normal (Figs. 40–41). Probably these result from the activity of mesenchyme cells which under normal conditions are inhibited in development. (Child, '16*a*.) (The possibility of stimulation of calcium metabolism by radiation should also be considered.)

Long exposures to radiation (7 minutes under Tungsten bulb, or 90 minutes in sunlight) produce some differential inhibition in unstained eggs (Figs. 42–47). In our previous work with activation of Echinoderm eggs by photodynamic action it was found that strong illumination frequently induces membrane formation, especially in eggs having more than the usual quantity of pigment, echinochrome. (Lillie and Hinrichs, '23.)

Two-day larvæ show greater effects of radiation than do eight-day larvæ, in most cases, indicating some degree of slow regulation and general recovery.

E. Experiments with Sperm.—Much work has been done in producing developmental modifications by subjecting one or the other germ cell to the deleterious action of chemicals (mostly anesthetics) and physical agents (particularly X-ray and radium) before fertilization. More work along these lines is contemplated with visible radiation following sensitization and a discussion of the literature will be reserved for a later paper.

It seems interesting to note, however, in connection with the work on differential developmental modifications, that eggs fertilized by sperm treated photodynamically (see also Bohn and Drzewina, '23 and Hertwig, '20), and eggs treated photodynamically before fertilization with normal sperm, develop into larvæ showing typical differential effects (Figs. 49–63).

Long exposures of sperm produce differential inhibition in the fertilized egg (Figs. 49–53, 55, 56), while shorter exposures produce a less harmful effect on the sperm, the resulting embryo shows indications of differential acclimation (Figs. 58–63).[1]

Discussion.—Concerning the action of a sensitizing dye like eosin, it is interesting to recall some of the conclusions reached by other experimenters,[2] namely; 1. The fundamental observations of von Tappeiner, '09, Hertel, '05, and Leo Loeb and his students, '07*a*, *b*, and the more recent evidence of Metzner, '21, '23, Schanz, '18, '21, Lillie and Hinrichs, '23, Hinrichs, '24, and Pereira, '25, have conclusively shown that eosin solutions, except in high concentrations depend for their toxicity on the presence of light, and that the resulting photodynamic action is probably primarily a surface effect (Lillie and Hinrichs, '23, and Pereira, '25).

2. Light to be effective must be absorbed (Grotthus-Draper Law), also see Hausmann, '23, p. 7. Ultraviolet radiation is directly absorbed by living protoplasm, primarily at the surface (Lillie and Baskervill, '22*a*, *b*). Visible radiation in the presence

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[1] The outline sketches on Plates I. and II. were made with a Bausch and Lomb euscope at intervals during development, and represent a magnification of about 60 diameters.

[2] For a complete review, see Dubois, '14, Hausmann, '23, and Bayliss, '20.

of a sensitizer like eosin appears to act primarily at the surface, as for example in membrane elevation in *Asterias* eggs (Lillie and Hinrichs, '23).[1] This effect may later be transmitted to adjacent regions (Hinrichs, '24, Henri, '12, Schleip, '23).

3. The action of light is a differential one (Bovie, '24, Hinrichs, '24, '25). This is evident from the fact that disintegration in lethal doses of radiation in the two spectral regions tested, ultraviolet and the blue-green region of the visible, follows an anteroposterior gradient along the axis of the body of lower organisms or embryos.

4. On the basis of such differential susceptibility to radiation, it has been possible to modify the development of eggs of *Fundulus* by exposure to ultraviolet radiation (Hinrichs, '25, unpublished). In the resulting embryos, those regions of the body which have the highest rates of physiological activity are the first to be modified. Accordingly forms appear in which the central nervous system, the heart and circulatory system, or the developing tail region are abnormal. Such effects may be produced by exposures made during the first ten minutes after fertilization, indicating that at this early stage physiological differences are already present which determine differences in susceptibility, *i.e.*, such differences exist even before there is any morphological differentiation visible.

5. Modification of development in *Arbacia* eggs is likewise dependent on such early differences in susceptibility. Child ('16*a*) has been able to produce developmental modifications with chemicals, and the results with radiation (see Child, '24*b*, footnote, p. 109) give further evidence, (1), of the non-specific nature of such susceptibility differences, and (2), of the dependence of these differences on quantitative differences along the physiological axes. Obviously there can be nothing specific, for example, in the inhibition of development of the oral lobe region by two such widely different agents as KCN on the one hand, and ultraviolet radiation, or visible radiation following sensitization on the other.

A region of the body which has a relatively high rate of physiological activity, as for example, the oral lobe region, is very intimately dependent on its environment for the continuance of its life processes. The more active such processes are, the more quickly will such a region register an incompatibility in its relation to its environment. Consequently, large doses of chemical or physical agents will produce a gradient of death and disintegration progressing from regions of high metabolic rate to those of lower rate; while in the case of sublethal doses of these agents, the result is an interference (temporary or permanent), with the normal growth and developmental capacities of the same region, the degree of interference being in general proportional to the intimacy of dependence of such regions on the environment.

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[1] Loeb, '07, Shippen, '07, and Pereira, '25, have found that eosin stains only dead cells. In other words, destruction of the surface film precedes cell cytolysis and subsequent staining by eosin.

As stated above, experiments in which normal eggs were fertilized by sperm which had been photodynamically treated, gave results of a similar kind. Since the embryo develops from a zygote, the sperm component of which is responsible for the bringing in of a protoplasmic system which is toxic for normal development of the egg protoplasm, and which remains a part of the developing embryo, there is obviously no result possible other than inhibition or acclimation. And since the developing embryo becomes differentially modified, it appears that the egg at the time of fertilization by the injured sperm, already shows evidence of the difference in susceptibility of its various parts.

Whatever the direct effect of light on living protoplasm, whether it be electron emission (Clark, '22) or heat sensitization (Bovie, '13, '23), there is certainly a fundamental relationship between the effectiveness of its action and the degree of physiological activity of the protoplasm acted upon. In general, the degree of physiological activity and the rate of oxygen consumption parallel each other, and since light action requires oxygen for its maintenance (under conditions of the experiment), it seems likely that there is some connection between the degree of effectiveness of light action and the rate at which the tissue consumes oxygen. (Further experimental data are necessary for the affirmation of such a premise.)

Of the dyes used, eosin and benzoflavine are adsorbed by the cell surface while neutral red and methylene blue are intra vitam dyes. Aside from their photodynamic properties, the latter two have shown themselves to be more toxic in comparable concentrations than eosin and benzoflavine even in the dark. (See also MacArthur, '24.) That they also possess photodynamic properties is evident from the fact that their toxicity, in weak concentrations, is augmented in the presence of light. (See Cooke and Loeb, '08, Bohn and Drzewina, '23, O. Hertwig, '12.) The present experiments have shown that methylene blue in comparable concentrations is about thirty times as toxic as eosin. An exposure of approximately fifteen times the duration is required to produce the same degree of inhibition with eosin as with one half the concentration of methylene blue. This is to be related to the position of the absorption band of methylene blue, which is near the region of maximum emission in the energy spectrum.

Eosin and benzoflavine have long been known as photodynamic sensitizers. Benzoflavine was found by Viale, '19, to be only slightly effective. On the other hand, eosin has been most commonly used as a sensitizer.[1] Eosin absorbs in the blue-green region, neutral red in the blue and blue-green, methylene blue in the orange and red, and benzoflavine in the azure-violet of the visible spectrum. Sensitization with these dyes makes tissues sensitive to the action of rays of a wave-length corresponding to the absorption band of the particular sensitizer used.

In comparing the relative effectiveness of the various light sources, it may be noted that with 1/10,000 eosin (30 minutes' staining), exposure to radiation from the photographic bulb was 90/7 times as effective in producing 100 per cent. inhibition as exposure to sunlight. This estimate is based on a ratio of the periods required in each case to produce inhibition in all eggs. Temperature was controlled.

Conclusions.—These experiments, in which the embryonic development of Arbacia was modified by means of visible radiation following sensitization, offer further evidence for the following:

1. The effectiveness of photodynamic sensitization depends upon (A) exposure of the sensitized system (in this case, fertilized eggs) to radiation of sufficient intensity and duration (dosage),

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[1] See Ledoux-Lebard, '02, Hertel, '05, von Tappeiner, '07, Loeb, '07*a*, *b*, Cooke and Loeb, '08, '09, O. Hertwig, '12, Schanz, '18, Clark, '22, Metzner, '21, '23, Lillie and Hinrichs, '23, Viale, '21, '23, '24, Hinrichs, '24, and Pereira, '25.

which in order that it may be absorbed and so become effective, must have, (*B*) a wave-length range including that of the absorption band of the particular sensitizer used.

2. The susceptibility to visible radiation following sensitization with the following sensitizing dyes (eosin, benzoflavine, neutral red, and methylene blue) is differential, *i.e.*, regions of high physiological activity are first to be modified in their development. Differential inhibition, recovery, and acclimation have been produced in this way.

3. It is possible to produce these effects (as with *Fundulus* and ultraviolet radiation) by exposure during the first few minutes after fertilization, indicating an early difference in susceptibility of various regions of the egg. Differential modification is also obtained by fertilizing normal eggs with photodynamically treated sperm.

4. These experiments confirm the view of the nonspecific and quantitative nature of susceptibility relations along the body axis.

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Explanation of Plate I.

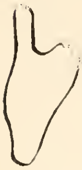
Figs. 1–3. Young plutei showing successive stages of early disintegration by photolysis. Lateral view, Fig. 2. Aboral view, Fig. 3.

Figs. 4–6. Normal three-day plutei. Fig. 4, Unstained, developed in dark. Fig. 5, Stained, developed in dark. Fig. 6, Lateral view of Fig. 5.

Figs. 7–22. Differential inhibition. Figs. 7–8, lateral view. Aboral arms and oral lobe slightly reduced. Figs. 9–11. Approximation of arms indicating inhibition of development of the median anterior region. Figs. 12–13, Complete reduction of median anterior region. Single median arm. (Figs. 7–13—Two-day larvæ. For normals see Fig. 48.) Fig. 14. Three-day larva. Union of two arms anteriorly, due to the reduction of the median anterior region. Figs. 15–16. More complete inhibition of development. Figs. 17–21. Exogastrulæ, indicating a difference in susceptibility to growth-inhibiting influences. Endoderm cells have continued to grow at the expense of ectoderm cells. Fig. 21. The widely diverging aboral arms indicate a secondary differential recovery of the median anterior region.

Figs. 23–27. Differential recovery. The anterior median region has recovered from a temporary inhibition and has regained and finally surpassed its normal growth rate. Oral lobe is over-developed. (Fig. 26. The angle of divergence between the aboral arms is much greater than normal.)

Figs. 28–30. Differences in degree of action of visible radiation. (*A*) Exposed without previous staining, Fig. 28. (*B*) After staining, Figs. 29, 30. The inhibitory effect is greater in *B*. Both *A* and *B* were exposed for the same period of time.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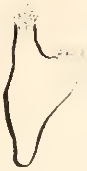
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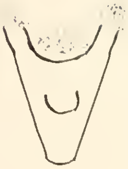
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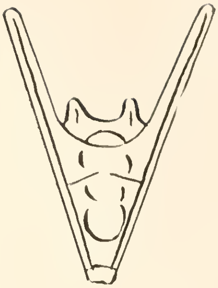
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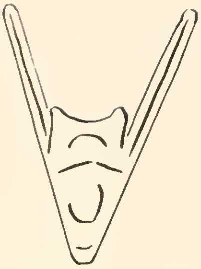
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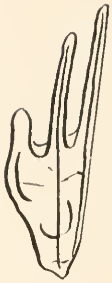
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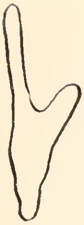
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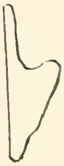
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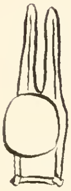
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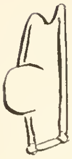
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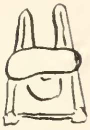
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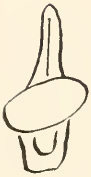
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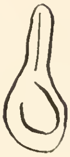
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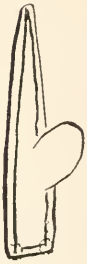
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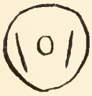
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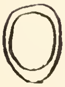
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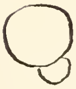
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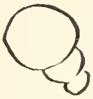
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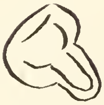
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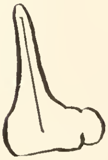
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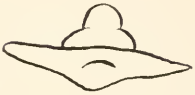
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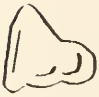
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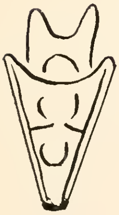
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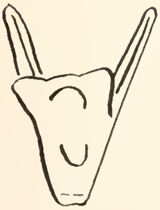
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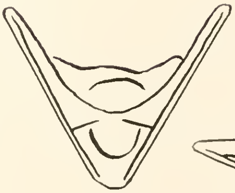
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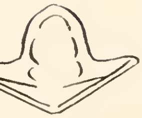
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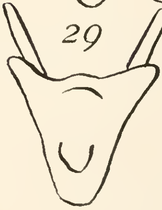
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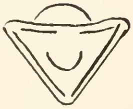
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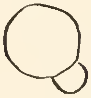
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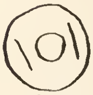
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M. A. HINRICHS.

Explanation of Plate II.

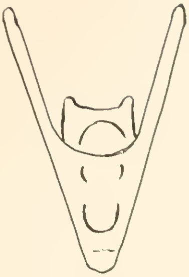
Figs. 31–32. Normal two-day larvæ.

Figs. 33–39. Effect of development in more concentrated solutions of sensitizing dyes in the dark. Figs. 33–36 show differential inhibition. Figs. 37–39 show differential acclimation. (Figs. 33, 34, 37, 38, development in eosin solution in the dark. Figs. 35, 39, benzoflavine, dark. Fig. 36, methylene blue, dark.)

Figs. 40–41. Overproduction in the number of aboral arms. Fig. 40. Unstained sperm exposed to radiation from photographic bulb for 30 minutes × normal egg. Fig. 41. Stained sperm (stained in the dark for 30 minutes before fertilizing normal eggs.)

Figs. 42–47. Effect of light action alone. (No previous sensitization.) Figs. 42–45 show differential inhibition. Figs. 46, 47 show differential recovery. (Figs. 42, 43, 44. Two-day larvæ exposed to direct sunlight for 90 minutes, immediately after fertilization. No previous staining. Fig. 45. Two-day larvæ. Exposed to radiation from photographic bulb for 7 minutes. No previous staining. Figs. 46, 47. Six-day larvæ. Exposed to direct sunlight for 90 minutes. No previous staining.)

Figs. 48–63. Effect of fertilizing normal eggs with photodynamically treated sperm. Fig. 48. Normal two-day larva. Control. Fig. 49. Differential inhibition followed by slight differential acclimation. Normal eggs fertilized by eosin-stained sperm exposed to radiation from photographic bulb for one minute. Figs. 50, 51. Exogastrulæ produced as a result of fertilizing normal eggs with sperm, eosin-stained, and exposed as above for 4 minutes. Figs. 52, 53, 55, 56 show differential inhibition. (Fig. 55 is result of exposing eosin-stained sperm to diffuse daylight for 30 minutes. Figs. 52, 53, 56, are the result of a 10-minute exposure of sperm before fertilization.) Fig. 54. General recovery. Appears normal. Normal eggs fertilized with eosin-stained sperm which had been exposed to radiation from photographic bulb for 1 minute. Fig. 57. General recovery. Stained sperm had been exposed for 30 minutes to radiation from bulb. Figs. 58–63. Differential acclimation. Fig. 58. Stained sperm exposed for one minute. Radiation from bulb. Figs. 59, 60. Result of 4-minute exposure. Figs. 61–63. Ten-minute exposure.



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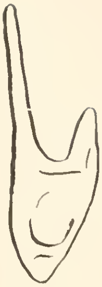

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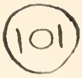

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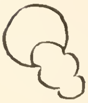

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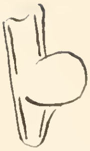

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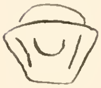

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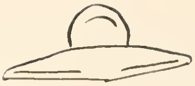

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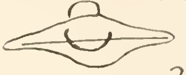

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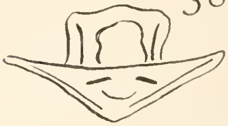

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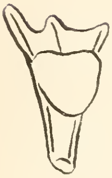

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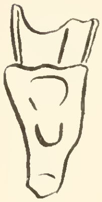

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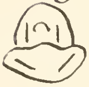

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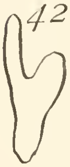

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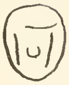

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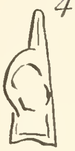

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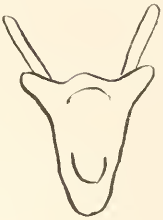

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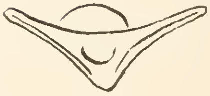

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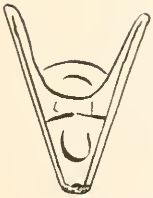

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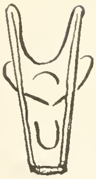

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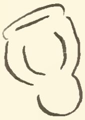

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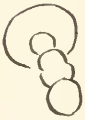

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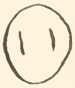

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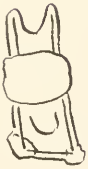

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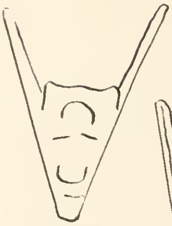

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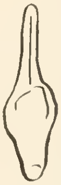

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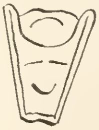

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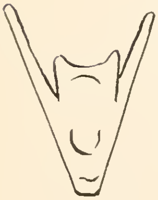

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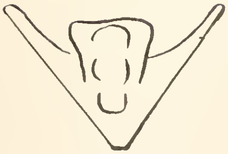

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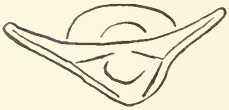

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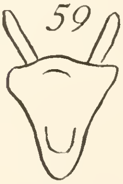

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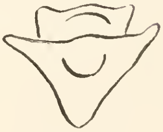

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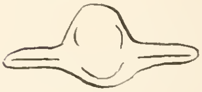

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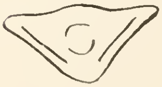

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M. A. HINRICHS.

FAUNA OF PENIKESE ISLAND, 1923.

(Edited by R. E. Coker.)

Introduction.

On the occasion of the fiftieth anniversary of the founding of the Agassiz laboratory on Penikese, a biological reconnaissance of the island was undertaken jointly by botanists and zoölogists from the Marine Biological Laboratory and the Fisheries Biological Station of the United States Bureau of Fisheries at Woods Hole, Mass. The studies of the flora have been published under the editorship of Professor I. F. Lewis in *Rhodora*, Vol. 26, Nos. 310, 311 and 312, 1924. The zoölogical collections and studies were made by the workers to be named hereafter, while the arrangement of the report for publication has been entrusted to the present editor.

With regard to the fauna, as well as to the flora, the collections were made by large parties, but chiefly on one day in each case. Practical considerations made it necessary for the zoölogists to restrict attention to the larger animals existing above the tide line, that is to say, to the non-microscopic animals living on the land or in the small freshwater and brackish ponds that are described by Miss Shaw in the report on Flora. Notwithstanding the number of collectors and the varied interests and experiences represented by them, the time spent on Penikese imposed an obvious limit to the exhaustiveness of the collections, especially since the fauna of the island is comprised largely in the group of Arthropods, so many of which are seasonal in appearance.

In the following list are the names of the persons comprising the zoölogical collecting expedition to Penikese Island, August 7, 1923, with the names of the respective institutions with which they were permanently engaged. The abbreviation "M.B.L." for Marine Biological Laboratory or "U.S.F.L." for United States Fisheries Laboratory, is used to denote the local laboratory to which each was temporarily attached during the summer of 1923.

F. E. Chidester, West Virginia University. U.S.F.L.
R. E. Coker, University of North Carolina. U.S.F.L.
Arch E. Cole, Northwestern University. M.B.L.
Manton Copeland, Bowdoin College. M.B.L.
J. A. Dawson, Dalhousie University. M.B.L.
S. R. Detwiler, Harvard University. M.B.L.
Samuel W. Geiser, Washington University. M.B.L.
Robert A. Goffin, U. S. Bureau of Fisheries.
Benjamin Grave, Wabash College. M.B.L.
Caswell Grave, Washington University. M.B.L.
George M. Gray, Marine Biological Laboratory.
Walter N. Hess, De Parmo University. M.B.L.
Hoyt S. Hopkins, Baylor Medical College. M.B.L.
Ondess L. Inman, Antioch College. M.B.L.
Ivey F. Lewis, University of Virginia. M.B.L.
Edwin Linton, Washington and Jefferson College and University of Georgia Medical Dept. U.S.F.L.
S. O. Mast, Johns Hopkins University. M.B.L.
C. E. McClung, University of Pennsylvania. M.B.L.
Everett C. Myers, Johns Hopkins University. M.B.L.
James T. Penney, University of North Carolina. U.S.F.L.
Albert M. Reese, West Virginia University. U.S.F.L.
A. H. Sturtevant, Carnegie Institute of Washington. M.B.L.
Henry C. Tracy, University of Kansas. U.S.F.L.
Arthur D. Whedon, North Dakota Agricultural College. M.B.L.
H. L. Wieman, University of Cincinnati. M.B.L.
Charles B. Wilson, Massachusetts State Normal School, Westfield. U.S.F.L.
John E. Wilson, Student Harvard Law School. U.S.F.L.
F. E. Wood, Illinois Wesleyan University. M.B.L.
Lorande Loss Woodruff, Yale University. M.B.L.
Donnell B. Young, Carleton College. M.B.L.

The following junior members of the expedition rendered helpful service in collecting:

Robert E. Coker, Jr.
Frederick C. Copeland.
Preston S. Copeland.

The party was conveyed from the Fisheries Laboratory at Woods Hole to Penikese Island by the U. S. Fisheries Steamer *Pharalope*. Collecting apparatus of every character as available was taken; there were seines, townets, dipnets, butterfly nets, sweeping nets, spade, rakes, gun, dipper, spoon, rat traps, mouse traps, etc., with necessary reagent for killing and preserving, and containers of various sorts, besides maps, labels, salinometers and cameras. The methods employed may be inferred from the apparatus taken.

It was originally contemplated to report the collections by station and habitat, but the character of the fauna of the island to which reference is made on page 25 below, made it evident that no particularly useful purpose would be served by adhering to a plan that would have both complicated the study and greatly extended the report.

Penikese Island. Northwest part, looking north from Pond 2-B.
(See footnote to p. 27.)

The study and identification of material was done in part by certain members of the collecting party, and in part by specialists associated with the United States National Museum and the Boston Society of Natural History; cordial acknowledgments are due to those institutions for the assistance that was so freely rendered. Appropriate credit to individuals is given in connection with the lists of species beginning on page 26, but in this place particular mention should be made of the following persons as the virtual authors of the systematic portion of this report: Messrs. C. W. Johnson (insects of several orders), A. H. Sturtevant (Diptera), C. B. Wilson (Odonata, aquatic Hemiptera, and aquatic Coleoptera), C. E. McClung (Orthoptera), Manton Copeland (Lepidoptera—butterflies), J. H. Emerton (Arachnida), Ondess L. Inman and Everett C. Myers (Birds).

Pond 1-*D*. (See footnote to p. 27.)

The general character of the island and its ecological features as regards the vegetation have been described by Lewis and Miss Shaw in the report previously mentioned. While the vegetational environments are of interest in connection with the consideration of the fauna, it would be superfluous to restate here the data to be found in that publication, especially in the section on "Ecology of Penikese" by Miss Margaret F. Shaw. It is necessary to give only a single quotation from Lewis (below) and to say that the small rocky island is practically devoid of trees, and, except where denuded of all vegetation in some of the nesting grounds of terns, is given over to grasses and other small plants, with rarely a low growing shrub or willow.

"The island Penikese is a remnant of the terminal moraine now seen in the Elizabeth Islands. It is about two thirds of a mile long and half as broad, with a broadly spatulate point for convenience of reference called Tub Point, extending further to the east for another third of a mile. Its contour is dominated by low hills on the main body of the island, with depressions here and there which may be ponds of a rather temporary character (map).

"The original vegetation, like that of neighboring islands, is said by Jordan to have been of a forest type, with pitch pine red cedar, red maple, shagbark, shadbush, poplar birch, hornbeam, and two or three species of sumac. In 1873 there was 'no trace left save the rotten roots of a solitary beech stump and a few branches of red cedar and red maple (?) found buried in the muck of a small swamp.' Cutting of the original timber had been followed by the introduction of sheep, which remained on the island until about 1910. From 1905 to 1921 Penikese was used by the State of Massachusetts as the site of a leprosarium. After the removal of the lepers in 1921 the island has been uninhabited except for the caretaker and his wife.

Pond 2-*D*. (See footnote to p. 27.)

"Jordan says that his 'list may have a special interest to future students, and also a general interest to botanists, as showing which plants survive a prolonged struggle for existence against grass and sheep.' If the State retains possession of Penikese and establishes a bird sanctuary there, the continued history of the development and succession of plants and animals cannot fail to be of interest and importance. Surveys at stated intervals will give precise information about natural succession under the prevailing climatic and edaphic conditions, and will furnish data on the means of dispersal of species in this region." [1]

A physical feature of little interest to the botanists but offering a relatively important habitat for animals consists in the low stone fences that have been built in somewhat rambling fashion over the hills of the main island. Though now falling down in places, these walls harbor numbers of spiders, isopods and insects. The old houses and sheds are likewise inhabited by many insects and spiders.

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[1] Lewis, I. F., "The Flora of Penikese, Fifty Years After," *Rhodora*, Vol. 26, No. 310, pp. 182–4, October, 1924.

Earlier Collecting on Penikese.

Although Penikese Island has been visited by naturalists from time to time during the past fifty years, only one paper has been encountered that deals with the fauna above the tide line. It may readily be understood why the pioneers of Agassiz's school had little time for study of the land fauna, engrossed, as they then were, with the revelations of marine life. Yet it seems worth while to quote in full the following communications from members of the original laboratory written fifty years after.

"I do not think that any one at Penikese listed the land snails, insects or other land animals except birds. We were busy with the novelties of the sea. C. O. Whitman gave most of his time to the birds, and I think had a list. F. H. Snow knew birds also. I made no formal list of the fishes, though I began my studies on them there. I could give an approximate record now. I listed also the Algæ, as well as the land plants. Walter Faxon recorded the marine Crustacea, and I made a list of the mollusks. Whether it is in existence now, I do not know. Time works havoc with manuscripts.

"Very sincerely yours,
"David Starr Jordan."

"I do indeed thank you for the pictures of Penikese and the laboratory at Woods Hole. I regret exceedingly that I cannot help you in the matter of Flora and Fauna of that Island. I was too busy with my lecture work to do more than that. The terns' nests with eggs were very abundant and a little work was done on its embryology.

"Faithfully yours,
"Edw. S. Morse."

Information of similar purport has been given me by Professor Cornelia M. Clapp, and Mr. Samuel E. Cassino, others who worked in the old laboratory on Penikese.

It is of particular interest that the only species described from Penikese (of which record has been found) is one of those relatively rare forms of crustacea, the branchiopods, that it should bear the name of the great teacher, and that the description should have been published in the second and final year of the original laboratory. The species is *Eulimnadia agassizii* Packard (1874);[1] it seems never to have been collected elsewhere.

In later years the island has been visited at various times by collectors and specimens have been deposited in several museums; but such collecting was done incidentally or in course of trips to various islands, and, almost invariably, without special publication. An exception is found in the paper by A. P. Morse entitled "Notes on the Orthoptera of Penikese and Cuttyhunk," appearing in *Psyche*, Vol. 7, p. 179, December, 1894.[2]

Among other naturalists who have submitted specimens from Penikese to various museums are J. P. Moore, Manton Copeland . Alexander Forbes, and J. T. Nichols. The following notes are quoted from a letter of Dr. Moore to the editor; "In 1892 and again in 1895, I collected in a small pond on Penikese two species of leeches, *Helobdella stagnalis*, and *Erpobdella punctata*. A few years later I again visited the island and think that I found leeches, but cannot find the record. Leeches are so easily carried by the aquatic birds that introduction is readily accomplished and one may expect to find other species. Even if all of the leeches should be destroyed by a prolonged period of drying of the small pools, the same or other species could be quickly reëstablished. . . . There are probably other species of Oligochæta in addition to the three named in your list. I know that I found *Enchytræus albidus* (= *Halodrilus littoralis*) on the strand and one of the small Naidæ in fresh water but these records also are not available just at present."

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[1] Packard, A. S. In Sixth Annual Report of the Peabody Academy of Science, Salem, Mass., 54, 1874.

In a later publication Packard says:

"About 100 females, mostly with eggs, occurred in a small pool of fresh water on Penikese Island, Buzzard's Bay, Aug. 27, 1873, collected by Mr. Walter Faxon. Upon examining the pool the following July or August (1874), the young, about a line in length, were found, but the pool subsequently dried up." (Quoted from "A Monograph of the Phyllopod Crustacea of North America, with remarks on the Order Phyllocarida," 12th Annual Report of the U. S. Geological and Geographical Survey of the Territories (for 1878), pages 295–590; Washington, 1883.

[2] I am endebted to Mr. C. W. Johnson for reference to the paper on Orthoptera. The species collected on Penikese are listed in another place. Editor.

Discussion of Results.

As will later be shown in detail, animals of 216 species were taken and subsequently identified, and these, grouped by phyla and lesser subdivisions, are distributed as follows:

Number of Species of Animals Collected from Penikese Island, August, 1923.

Annelids	3	species
Mollusks	1	“ [1]
Arthropods:		
Crustacea	2	“
Myriapods	1	“
Insects	162	“
Arachnids	15	“
Vertebrates:		
Amphibia	1	“
Reptiles	1	“
Birds (actually resting on the island)	29	“
Mammals	1	“
Total	216	“

Insects by Orders:	
Odonata	3
Orthoptera	8
Hemiptera (terrestrial 20, aquatic 6)	26
Neuroptera	2
Lepidoptera	13
Diptera	47
Coleoptera (terrestrial 30, aquatic 24)	54
Hymenoptera	9
Total number of species of insects	162

It is worth while to allude to certain groups that are not represented in the list. Although a thorough search was made for fish in the brackish and freshwater ponds, none were found. Since isolation is not usually sufficient to account for the want of small fish in ponds, even such as may occasionally become nearly dry, the absence of such forms is doubtless to be attributed to the abundance of fish-eating birds on the island. It is notable in this connection that the amphibia were represented by a single species, the common toad, the least attractive member of the class to carnivorous animals. Among reptiles, too, turtles and water snakes must be extremely rare, if present at all, the only reptile taken being the common garter snake, of which several specimens were obtained both by the botanists on July 24, and by the zoölogists on August 7. Toads were also taken on both days. It appears that previous collectors interested in the herpetology of the island have found only the toad and the garter snake. It is surprising that aquatic crustacea were not found. Not even were copepods encountered in net collections from the small ponds. Perhaps in this respect, as in others, the results would have been different at another season.

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[1] An aquatic gastropod lost before identification.

Viewing the fauna of the island, as a whole, we observe that it is largely arthropodan—insect, arachnid and myriapod. Exclusive of arthropods and birds, we found but 4 species of invertebrates (3 earthworms and 1 gastropod) and 3 of vertebrates (an amphibian, a reptile, and a mammal). The invertebrate fauna would have been greatly extended by giving consideration to minute forms of animal life; but the vertebrate fauna was doubtless fully covered, except as to birds, which are migratory and seasonal. The apparent relative poverty of the island in nearly all forms of animal life (as regards number of species) may be attributed in great part to the very limited variety of environments, as afforded by plants and physical conditions.

An outstanding feature of the fauna of Penikese Island is its composition (almost entirely) of forms of very general distribution for the latitude. This, of course, is to be expected, since there are no known conditions upon the island to suggest the probability of the occurrence of unusual forms of life. In such a place we must seek not to find a fauna of special types, but rather to ascertain which of the common types of animal life encounter there the conditions favorable or requisite for their existence. Nevertheless, Penikese happens to be the place of collection of at least one specific type. (See p. 23 above.)

It is hoped that, quite apart from any interest which might attach to this survey from its relation to the historical place and occasion, it will serve a useful scientific purpose as a basis of comparison with future conditions. It would be desirable for collections to be made at all seasons, under various weather conditions and in different years. Penikese Island has recently been set aside by the State of Massachusetts as a Bird Sanctuary, and placed under the Board of Conservation who are interested in fostering both the birds and the insect life of the island. The entire aspect of the insect fauna of the island may change materially at any time in consequence either of the invasion of new species adapted to present conditions but not yet arrived, or as a result of changing conditions of vegetation upon the island, such, for example, as would follow upon renewal of grazing or upon natural or artificial reforestation. In event of such change it will fortunately be possible, to some extent, to compare new and old conditions with respect to both plants and animals, and thus to study problems of coördination between the two kingdoms. We have, therefore, thought it a proper function of the introductory part of this report to direct attention to the forms that appear to be lacking, as well as to those that were found. Needless to say, future comparisons must not omit from consideration the season of the year and the precedent meteorological conditions such as are of record elsewhere than in this paper.

Animals of Land and Ponds,

Penikese Island, August, 1923.

ANNELIDS.

(Identification by U. S. National Museum.)

Lumbricus terrestris Linnæus.

One adult and one apparently immature example taken near red house.

Helodrilus calignosus trapezoides (Savigny).

One example taken at same place.

Helodrilus tenuis (Eisen).

Two examples taken with preceding species and one in southern part of island.

MOLLUSCS.

(Gastropod from pond not identified.)

ARTHROPODS.

Crustacea.

(Identification by U. S. National Museum.)

Oniscus asellus Linnæus.

Porcellio rathkei Brandt. Abundant under boards and logs and in stone fences.

Myriapods.

(Identification by R. V. Chamberlain, Museum of Comparative Zoölogy, Cambridge, Mass., through U. S. National Museum.)

Lithobius forficatus Linnæus.

Insects.

Odonata.[1]

(Identification by C. B. and J. E. Wilson.)

Anax junius (Drury). Pond 1–*B*; three nymphs.
Pond 2–*B*; four nymphs.
Pond 1–*D*; seven nymphs.

Celithemis eponina (Drury); seen flying about in two places.

Ischnura verticalis (Say); Pond 2; two adults.
Pond 2; four adults.

Orthoptera.

(Identification by C. E. McClung.)

Gryllidæ.

Gryllus assimilis.

Nemobius vittatus Harr.

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[1] In reports on aquatic insects (Odonata, Hemiptera, Coleoptera) the several small ponds are distinguished by letters and numbers, as follows: The two small water-holes near the northwest corner of the island are designated 1-*B* and 2-*B*, 1 being southwestward of 2; 1-*C* (Cattail Pond) is on the eastward side of the island near the keeper's house; near the southerly tip of the eastward extension of the island (Tub Point) are two small ponds, 1-*D* and 2-*D*, 1 being northeastward of 2. The largest pond, that near the southerly point of the main island, known to the collectors as South Pond (*A*) was decidedly brackish and yielded only one aquatic insect. The specific gravities (uncorrected for temperature) and the temperatures of the waters of these ponds were as follows: South Pond, 1.015 (20° C.); 1-*B* and 2-*B*, 0.99 (21° C.); 1-*C*, 0.99 (20° C.); 1-*D*, 1.0018 (23° C.); 2-*D*, 1.005 (22° C.).—EDITOR.

Locustidæ.

Conocephalus fasciatus De G.

Orchelimum vulgare Harr.

Acrididæ.

Melanoplus femur-rubrum De G.

Melanoplus atlanis Riley.

Chorthippus maculipennis Scudd.

Dissosteira carolina Linn.

Generally distributed, localized only by distribution of food plants.

The following list of Orthoptera appeared under the title "Notes on the Orthoptera of Penikese and Cuttyhunk," by A. P. Morse. (*Psyche*, Vol. 7, p. 179. Dec. 1894.)[1]

SPECIES COLLECTED ON PENIKESE.

Acrididæ.

Stenobothrus æqualis Scudd. Common.

Stenobothrus maculipennis Scudd. Common.

Chortophaga viridifasciata De G. Scarce.

Dissosteira carolina Linn. Common.

Psinidia fenestralis Serv. Scarce.

Melanoplus atlanis Riley. Scarce.

Locustidæ.

Xiphidium (*Conocephalus*) *fasciatum* De G. Common.

Gryllidæ.

Nemobius vittatus Harris. Common.

Hemiptera (Heteroptera).

(Identification by C. W. Johnson, Boston Society of Natural History.) (See also "Aquatic Hemiptera" below.)

Pentatomidæ.

Cœnus delius Say.

Coreidæ.

Harmostes reflexulus Say.

Lygæidæ.

Cymus luridus Stål.

---

[1] Note by courtesy of C. W. Johnson.

Nabidæ.

Pagasa fusca Stein.
Nabis propinquus Reut.
Nabis ferus Linn.

Miridæ.

Teratocoris discolor Uhl.
Pœciloscytus basalis Reut.
Lygus pratensis Linn.
Onychumenus decolor Fall.

Saldidæ.

Saldula interstitialis Say.

Hemiptera (Homoptera).

(Identification by C. W. Johnson, Boston Society of Natural History.)

Cercopidæ.

Philænus leucophthalmus Linn. var. *ustulatus* Fall.
Philænus lineatus Linn.

Cicadellidæ.

Helochara communis Fitch.
Dræculacephala mollipes Say.
Dræculacephala minor Walk.
Acucephalus albifrons Linn.
Deltocephalus simplex Van D.

Fulgoridæ.

Liburnia pellucida Fabr.
Liburnia campestris Van D.

Aquatic Hemiptera.[1]

(Identification by C. B. and J. E. Wilson.)

Arctocorisa interrupta (Say); Ponds 1–*D* and 2–*B*; three specimens in all.

Belostoma flumineum Say; Pond 1–*B*; one nymph, identification doubtful.

Corixa verticalis Fieber: Pond 2–*D*; twenty-two specimens.
Pond 1–*D*; twenty specimens.
Pond 1–*B*; one specimen.
Pond 2–*B*; twenty specimens.
South pond; one specimen.

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[1] See footnote to page 27.

Buenoa margaritacea Bueno; Pond 1–*D*; one doubtful nymph.

Gerris marginatus Say; Pond 1–*B*; ten specimens; Pond 1–*D*; one nymph.

Microvelia borealis Bueno; Pond 2–*B*; one specimen.

Neuroptera.

(Identification by C. W. Johnson, Boston Society of Natural History.)

Hemerobiidæ.
  Hemerobius stigmaterus Fitch.
Chrysopidæ.
  Chrysopa oculata Say.

Lepidoptera—Butterflies.[1]

(Identification by Manton Copeland, Preston S. Copeland and Frederick C. Copeland.)

Pieris rapae Linnæus (9).
Euvanessa antiopa Linnæus (1).
Vanessa atalanta Linnæus (1).
Vanessa huntera Fabricius (1).
Junonia cœnia Hübner (4).
Heodes hypophlæas Boisduval (5).

Lepidoptera—Moths.

(Identification by C. W. Johnson through Mr. Copeland.)

Hadena lignicolor Guenée (1).
Drasteria erechtea Cramer (3).
Drasteria crassiuscula Haworth (7).
Pyrausta fissalis Grote (1).
Crambus hortuellus Hübner (18).
Argyria nivalis Drury (1).
Ancylis Hübner sp? (1).

Diptera.

(Identification by A. H. Sturtevant, whose report is transmitted with the letter that is quoted immediately following.)

"I am enclosing the list of Penikese Diptera. There are 47 species on it—and these are all that I am able to identify with certainty. I should judge that there are about 30 additional species in the material, including the most conspicuous Dipteran encountered on the island, the mosquito, which apparently belongs in the genus Aedes, but which I have not included because the species remains very uncertain.

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[1] Figures in parentheses denote numbers of examples taken.

"There is one record that seems never to have been published as American, though the insect is so common that I suspect several people must have identified it correctly before—I have myself had the necessary information for two years now. One or two of the other records are of some interest from the point of view of geographical distribution, and that of *Muscina pascuorum* is apparently the only one for 1923 as early as August. Since the species appears to have been introduced from Europe only within the past few years any such data about it are of value, I think."

Asilidæ.
  Asilus paropus Walker (Determined by C. W. Johnson).

Dolichopodidæ.
  Dolichopus albicoxa Aldrich.
  D. bifractus Loew.
  D. pugil Loew.
  D. splendidus Loew.

Lonchopteridæ.
  Lonchoptera furcata Fallen.

Syrphidæ.
  Allograpta obliqua Say.
  Eristalis tenax Linne.[1]
  Helophilus lætus Loew.
  Platychirus quadratus Say.
  Toxomerus marginatus Say.

Muscinæ.
  Muscina pascuorum Meigen.

Stomoxyinæ.
  Stomoxys calcitrans Linne.

Calliphorinæ.
  Calliphora erythrocephala Meigen.

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[1] Larvæ of this, or a very similar species were very common in samples of mud taken from the bottom of freshwater ponds.—R. E. C.

Lucilia cæsar Linne.
Lucilia sylvarum Meigen.
Pollenia rudis Fabricius.

Ortalinæ.
Chætopsis fulvifrons Macquart.
C. massyla Walker. These two species were taken in large numbers in the salt marshes, but no specimens were obtained of *C. ænea* Wiedemann and *C. apicalis* Johnson, which are the common salt marsh species of the mainland and of Naushon.

Trypaneinæ.
Euaresta bella Loew.
Trypanea abstersa Loew.
T. daphne Wiedemann.

Agromyzinæ.
Cerodonta dorsalis Loew.

Sepsinæ.
Sepsis violacea Meigen.
Themira minor Haliday.

Ochthiphilinæ.
Ochthiphila polystigma Meigen.

Chloropinæ.
Botanobia coxendix Fitch.
B. frit pusilla Meigen.
Chloropisca glabra Meigen.
Elachiptera costata Loew.
Melanochæta longula Loew.

Drosophilinæ.
Drosophila quinaria Loew.
Scaptomyza adusta Loew.

Ephydrinæ.
Dichaeta caudata Fallen.
Dimecœnia spinosa Loew.
Ephydra subopaca Loew.
Notiphila scalaris Loew.
Ochthera mantis DeGeer.
Pelina truncatula Loew.

Psilopa atrimana Coquillett.
P. flavida Coquillett.
Scatella dichæta Loew.

Borborinæ.

Leptocera atra Adams. Common and widespread.

Leptocera brachystoma Stenhammar. This species does not seem to have been recorded as American. It is common under eel-grass at high tide level at least throughout the Woods Hole region. I have compared my specimens with British material determined by J. E. Collin, Esq.

Leptocera lutosa Stenhammar. Common and widespread.

Borborus geniculatus Macquart.

Phycodrominæ.

Cœlopa parvula Haliday.

Coleoptera.

(Identification by C. W. Johnson, Boston Society of Natural History.)

Carabidæ.

Galerita janus Fabr.
Chlænius pennsylvanicus Say.
Harpalus caliginosus Fabr.
Harpalus compar Lec.
Harpalus pennsylvanicus DeGeer.
Harpalus herbivagus Say.

Staphylinidæ.

Ocypus ater Grav.

Silphidæ.

Necrophorus americanus Oliv.
Necrophorus marginatus Fabr.
Silpha lapponica Hbst.

Histeridæ.

Saprinus sp.

Cantharidæ.

Silis bidentatus Say.

Mordellidæ.

Mordellistena pustulata Melsh.

Elateridæ.
  Melanotus fissilis Say.
    Also a number of larvæ.
  Melanotus communis Gyllenhall (Identified by U. S. National Museum).

Dermestidæ.
  Dermestes vulpinus Fabr.

Nitidulidæ.
  Conotelus obscurus Er.

Phalacridæ.
  Stilbus apicalis Melsh.

Coccinellidæ.
  Hippodamia parenthesis Say.
  Coccinella novemnotata Hbst.

Tenebrionidæ.
  Blapstinus mæstus Melsh.
  Tenebrio obscurus Fabr.

Scarabæidæ.
  Aphodius fimetarius Linn.
  Xyloryctes satyrus Fabr.
  Phyllophaga? sp.? (larvæ).

Chrysomellidæ.
  Calligrapha elegans Oliv.
  Galerucella notata Fabr.
  Diabrotica duodecimpunctata Fabr.
  Phyllotreta sinuata Steph.

Curculionidæ.
  Brachyshynchus ovatus Linn.

Aquatic Coleoptera.[1]

(Identification by C. B. and J. E. Wilson.)

Acilius semisulcatus (Aubé); Pond 1–*D*; two specimens.
Agabus disintegratus Crotch; Pond 1–*B*; three specimens.
Berosus striatus (Say); Pond 1–*B*; two specimens.
Cœlambus impressopunctatus (Schall.); Pond 1–*D*; nine specimens.

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[1] See footnote to p. 27.

Cœlambus inæqualis Fabricius; Pond 1–*B*; six specimens.
Deronectes depressus Fabricius; Pond 1–*B*; ten specimens.
Dytiscus fasciventris Say; Pond 1–*C*; two specimens.
Hydrophilus obtusatus (Say); Pond 1–*B*; one specimen.
Haliplus ruficollis DeGeer; Pond 2–*B*; eighteen specimens.
Hydroporus americanus Aubé; Pond 1–*B*; three specimens.
Hydroporus modestus Aubé; Pond 2–*B*; twelve specimens.
Hydroporus niger Say; Pond 1–*B*; fourteen specimens.
Hydroporus proximus Aubé; Pond 1–*B*; two specimens.
Hydroporus stagnalis G. & H.; Pond 1–*B*; seventeen specimens.
Hydroporus tenebrosus Lecepède; Pond 1–*B*; one specimen.
Hydroporus undulatus Say; Pond 1–*B*; twenty specimens.
Octhebius foveicollis Lecepède; Pond 2–*D*; two specimens.
Philhydrus cinctus Say; Pond 2–*D*; fifty specimens.
Philhydrus nebulosus Say; Pond 1–*D*; one specimen.
Rhantus binotatus Harris; Pond 1–*D*; two specimens.
Rhantus bistriatus Bergst.; Pond 1–*B*; one specimen.
Thermonectes basilaris Harris; Pond 1–*D*; two specimens.
Tropisternus glaber Herbst; Pond 2–*B*; six specimens.
Tropisternus nimbatus Say; Pond 1–*C*; one specimen.

Hymenoptera.

(Identification by C. W. Johnson, Boston Society of Natural History.)

Ichneumonidæ.
  Therion morio Fabr.

Formididæ.[1]
  Myrmica scabrinodis Nyl. subsp. *schencki* Emery var. *emeryana* Forel.
  Lasius niger Linn. var. *americanus* Emery.
  Lasius (*Chthonolasius*) *brevicornis* Emery.

Halictidæ.
  Halictus provancheri Cress?

Megachilidæ.
  Osmia lignaria Say.

Apidæ.
  Bombus fervidus Fabr.

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[1] Identified for the Boston Society of Natural History by Dr. W. M. Wheeler.

Bombus americanorum Fabr.
Bombus bimaculatus Cress.

Arachnids.

(Identification by J. H. Emerton, through U. S. National Museum.)

Enoplognatha marmorata Hentz.
Epeira trivittata Keys. Many specimens.
Epeira pratensis Hentz. Immature.
Epeira stellata Hentz. Immature.
Argiope trifaciata Forskal. Young, several specimens.
Tetragnatha laboriosa Hentz.
Agalena naevia Walck. All young, half grown.
Dolomedes sexpunctatus Hentz. Young.
Lycosa helluo Walck. With egg cocoon.
Pardosa nigripalpis Em.
Pardosa glacialis Th.
Pardosa lapidicina Em.
Zelotes ater Hentz. Adult male and young.
Ariadne bicolor Hentz.
Phidippus multiformis Em.
Xysticus gulosus Keys.[1]

Birds.

Observed and identified by Ondess L. Inman and Everett C. Myers. (With confirmatory observations by others.)

Song Sparrow—*Melospiza melodia melodia.* Most common land bird.
Chipping Sparrow—*Spizella passerina passerina.* Few.
Seaside Sparrow—*Passerherbulus maritimus maritimus.* Common.
Sharp tailed Sparrow—*Passerherbulus caudacutus.*
English Sparrow—*Passer domesticus.* Fairly common about buildings.
Field Sparrow—*Spizella pusilla pusilla.* Few.
Vesper Sparrow—*Poocætes gramineus gramineus.*
Common Tern—*Sterna hirundo.* Most common.

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[1] Specimens identified by J. H. Emerton through Boston Society of Natural History.

Roseate Tern—*Sterna dougallii.* Fairly common.
Least Tern—*Sterna antillarum.* Rare.
Cory Shearwater—*Puffinus borealis.* Rare.
Herring Gull—*Larus argentatus.* Rare.
Laughing Gull—*Larus atricilla.* Rare.
Meadow Lark—*Sturnella magna.* Few (6).
Yellow Warbler—*Dendroica æstiva æstiva.* One pair.
Upland Plover—*Bartramia longicanda.*
Semipalmate Sandpiper—*Ereunetes pusillus.* Very common.
Little Green Heron—*Butorides virescens.*
Ruddy Turnstone—*Arenaria interpres morinella.* Two.
Red-wing Blackbird—*Agelaius phœniceus.* Common.
Spotted Sandpiper—*Actitis macularia.*
Least Sandpiper—*Pisobia minutilla.*
Killdeer—*Oxyechus vociferus.*
Yellow legs—*Totanus* sp.? Rare.
Woodcock—*Philohela minor.*[1] One.
Virginia Rail—*Rallus virginianus.*
Curlew—*Numenius* sp.?
Eve Swallow?—
Ringneck Plover—*Aegialitis semipalmata* (Semipalmate Plover).

Other Vertebrates.

Bufo fowleri—Common toad. 4 examples.
(Identified by Miss Doris M. Cochran, U. S. National Museum.)
Thamnophis sirtalis sirtalis—Common garter snake. 6 examples.
(Identified by Miss Doris M. Cochran, U. S. National Museum.)
Peromyscus leucopus noveboracensis (Fischer). Field mouse. One example taken in one of the traps left on the island. Specimen preserved by the kindness of the State caretaker on Penikese; identified by F. E. Wood.

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[1] Reported by D. B. Young.

THE CAUSE AND NATURE OF ENCYSTMENT IN *POLYTOMELLA CITRI.*

J. McA. KATER and R. D. BURROUGHS,

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Although encystment has long been known to play an important part in the life of most protozoa the cause and nature of the phenomenon has been the subject of relatively little careful experimental work. The evidence derived from the earlier work, slight as it was, together with the fact that cysts of protozoa are very resistant to drying, toxic substances, etc., has led the writers of text-books to speak of encystment as a passive response to adverse environmental conditions (Calkins 1909, Minchin 1912, Doflein 1916). In spite of the fact that such generalizations are quite common, there is little evidence that, on critical examination, cannot be interpreted otherwise than as mentioned above. The general confusion that has arisen from the conflicting data of almost every piece of work on this subject makes it desirable to give a brief historical review.

From the time that encysted protozoa were first observed in 1769 by Saussure until 1855 the few observations made on this subject did not deal at all with the cause of the process, but merely with establishing the relationship between cysts and the active forms to which they give rise. In 1855 Cienkowsky not only called attention to the fact that encystment is probably not universal among protozoa (as was supposed before that time) but also succeeded in producing encystment by a lengthy drying up of the medium.

After this date dessication was considered to be the principal, if not the sole cause of encystment, until the subject was attacked by Maupas and Fabre-Doumergue in 1888. The former produced encystment in predaceous ciliates by the deprivation of food, while the latter showed that drying up of the medium was not of paramount importance. By growing several ciliates in cultures placed in a moist chamber, he discovered that they regularly encysted. This author attributed the causal factors to putrefaction of the medium, resulting in the release of toxic chemical substances.

The contention of Maupas has received support from other workers. Among them Root (1914) concludes that lack of food is the cause of encystment in the suctorian Podophrya collini. Mast (1917) obtained similar results with *Didinium nasutum*. He says: "Encystment in Didinia can usually be induced by cutting off the food supply. But it frequently occurs when there is an abundance of food present and sometimes it does not occur when there is none." However, Mast and Ibara (1923) after some carefully conducted experiments decide that "They encyst more freely in cultures supplied with food than in those without food, and this probably is due to greater increase in numbers, resulting in greater accumulation of waste material in the one than in the other." Hogue (1915) observed that the limax amœba upon which she was working encysted in the presence of abundant bacteria, upon which it feeds, and that the cell body of the encysting amœba always contained numerous bacteria. In both this paper and a later one (1917) she expresses the opinion that accumulation of byproducts of metabolism and oxygen deficiency are the principal causal factors. She thinks that they operate by causing the amœbæ to lose the power of assimilation. Carter (1919) concludes that abundant food is necessary for encystment of *Amœba proteus*.

Kofoid and Swezy (1921) consider that encystment in the marine dinoflagellates is due to one of two causes, the need for protection during the period of multiple and binary fission, and the need for a quiescent period for the assimilation of a large food body that has been ingested. Hall (1925) assigns *Oxyrrhis marina* to the second group given by Kofoid and Swezy. In addition, referring to drop cultures sealed with vaseline, he says: "After six weeks, exclusively non-motile forms were observed; in these, no flagella could be detected in observation under the oil immersion objective, and in some cases a 'cyst wall' seemed to surround the flagellates." Since no food vacuoles were present, he thinks that these non-motile forms may represent cysts formed in response to unfavorable conditions. From his figures of cysts one would think that there should be no question of the presence of a cyst wall if one were there and it seems doubtful that they were really cysts.

Stolte (1922) reports that the ciliate *Blepharisma* encysts only in the presence of abundant food, and that large food vacuoles are present in those entering encystment. He also emphasizes the necessity for a relatively large number of *Blepharisma* in the culture before encystment is possible. This conclusion concerning a ciliate is quite in harmony with Kofoid and Swezy's for dinoflagellates.

Koffman (1924) suggests hydrogen-ion concentration as one of the essential factors in producing encystment in ciliates. He says: "The ciliates require ordinarily a definite P_h zone with a definite optimum, for their development. Below the minimum as well as above the maximum either initiates an encystment or the animals are destroyed."

In direct contradiction to the conclusions of the two preceding authors, we might mention the work of Brand (1923) who considers that, in the case of *Vorticella microstoma*, hunger, oxygen deficiency, and gradual drying up are the principal external factors for encystment. He relegates products of metabolism and chemical influence in general to a position of less importance.

Calkins (1915) found that *Didinium nasutum* regularly encysts entirely independent of adverse environmental conditions. However, encystment in this ciliate may not be entirely comparable to that of other forms, as a nuclear reorganization, similar to endomixis, occurs during the encysted phase. Fermor (1913) has described the same sort of reorganization in *Stylonychia pustulata*. With regard to the significance of encystment in *Didinium* Calkins says: "In addition to the casual encystment resulting from adverse environmental conditions, there is another form of encystment which involves more deep lying activities of the protoplasm," and again, "When the process is completed and the organisms emerge from their cysts they possess from five to seven times the vitality, measured by the division rate of the same race prior to encystment." In the face of this statement it is interesting to quote Mast (1917): "there was no evidence obtained indicating that conjugation or encystment has any effect on death rate, fission rate, or variation in fission rate. This would indicate that neither of these processes is a rejuvenating process, at least not in the sense in which Calkins has used the term."

The foregoing review certainly indicates that more experimental work is needed on this subject, not only to obtain a better understanding of the protozoan life cycle, but also for the elucidation of the general biology of the so-called "resting forms of protoplasm." With this in view we have undertaken the work recorded in the following pages. The colorless phytomonad, *Polytomella citri* was selected as the organism to be experimented upon for two reasons: (*a*) it encysts very readily, and (*b*) most of the previous work has been confined to ciliates and amœbæ and we thought it desirable to extend the experimental field to flagellates.

We wish to express our gratitude to Professor E. G. Conklin, not only for his criticism, but also for his interest and encouragement. Our thanks are also due to Professor E. Newton Harvey for some very valuable suggestions.

MATERIAL AND METHODS.

The culture medium used in all of the experiments described in the following pages was made by boiling timothy hay in distilled water, about ten grams of hay to a liter of water. Three liters was the quantity usually made at one time, and all of the cultures of any one experiment contained medium of a single making, thus eliminating the possible error due to variation in the medium. Test tubes served the purpose of containers. Approximately 15 cc. was the quantity of medium in each culture.

Attempts were made to determine the effect of (1) various temperatures, (2) hydrogen-ion concentrations, (3) metabolic by-products, and (4) food deficiency on the tendency to encyst.

(1) The different temperatures were chosen because of their availability; and in spite of the fact that there was considerable variation in some cases the results were sufficiently striking for the present purpose. No attempt was made to arrive at an optimum temperature within a narrow range, as this was not thought to be necessary.

(2) For the work on hydrogen-ion concentration the ordinary hay infusion was used and the P_h was altered by either sodium hydroxide or acetic acid. The hydrogen-ion concentration was determined by the colorimetric method.

(3) The problem of metabolic by-products was approached in two different ways: first, by filtering the cultures through a porcelain filter and replacing the medium with fresh hay infusion, and, second, by removing the fluid from a thriving culture, in the same way, and replacing with medium taken from an old culture in which there were none active, all being encysted. In the one case the by-products were kept at a minimum, in the other they were increased beyond normal.

(4) To obtain a low food content in the cultures the hay infusion was replaced with distilled water and various percentages of distilled water and fresh hay infusion.

It has become rather customary, in recent years, to carry on work of this kind on organisms that have all been derived from a single individual. By this method one avoids comparing different strains, but, on the other hand, the results are applicable only to that particular strain, which probably can never be duplicated. The organisms used in the present work were all derived from a single culture, not from a single individual. It was thought, at the beginning, that by running a great number of cultures conclusions could be arrived at that would be applicable to the species, and that minor variations could be smoothed out by amount of data. The remarkable correspondence in the different cultures of any one series has entirely justified this procedure. This agreement has been so great that it has been possible to give only one result for all cultures of a series, with only two exceptions.

The condition of the cultures, in respect to active forms, is designated by the following terms: *very poor*, *poor*, *fair*, *good*, and *excellent;* and, with regard to cysts by: *very few*, *few*, *some*, *many*, and *very many*. These indefinite terms will doubtless arouse criticism. To this we can only reply that the differences in both active and encysted forms in the different series was so striking in all cases that they could easily be placed in their proper class, and, secondly, that it is the relationship which is of importance and not the actual numbers.

Polytomella was associated with bacteria and yeast in all of the cultures.

EXPERIMENTAL.

Temperature.—The procedure determined upon was to eliminate temperature first and then carry on the remainder of the experiments at the optimum temperature. Accordingly, thirty-six cultures were seeded and twelve of these were kept at each of the following temperatures: 10°–11° C. (series *A*1), 21°–22° C. (series *A*2), and 22°–27° C. (series *A*3). Series *A*3 was kept in the laboratory, which explains the wide variation in temperature. At the end of nine days all cultures of the three series were in excellent condition. Those of series *A*1 showed no cysts, *A*2 contained few, while some were found in *A*3. In fourteen days *A*1 was in good condition with no cysts; *A*2 was in fair condition, with some cysts; and *A*3 was poor, with many cysts. In twenty-one days *A*1 was only fair and no cysts had yet appeared; *A*2 had reached the same condition that *A*3 was in on the fourteenth day and *A*3 now contained no active forms and many cysts. The active forms had entirely disappeared from *A*2 by the thirty-fourth day. These two series continued in the same condition for the remainder of the experiment. On the thirty-fourth day *A*1 was in poor condition, without encystment. From the thirty-sixth to thirty-ninth day there was no ice in the refrigerator and the temperature rose from 10° to 21° C. At the end of those three days all twelve cultures were in fair condition and a few cysts had appeared in five of them. On the thirty-ninth day ice was again placed in the refrigerator and the experiment continued for twenty-one more days. At the end of that time there were practically no active forms in any of the cultures and the few cysts that were in five of the cultures on the thirty-ninth day were still present, but the remaining seven cultures had died out without any encystment whatever.

The first attempt showed that the cultures kept at 35° C. would not develop. Consequently, to attempt to determine the effect of so high a temperature, cultures were permitted to develop in the laboratory for a few days and were then placed in the incubator. It was found that at 35° thriving cultures would gradually die out until, at the end of two days, they would have completely disappeared without the formation of a single cyst. A little later twelve cultures were seeded and six of these were placed in the laboratory, the other six at a temperature of 30° C. In two days all were excellent cultures and many cysts had appeared in the ones kept in the laboratory, while none had encysted at 30°. On the third day all were alike, fair cultures with many cysts. The reason for these encysting more quickly than those of Series *A* will be explained later.

A second set of temperature experiments was started some time after series *A*. Series *B*1, *B*3 and *B*4 were kept at the same temperatures respectively as *A*1, *A*2 and *A*3. Series *B*2 was kept at 15°–22° C. With only slight variation the results were the same in series *B* as in *A*. Observations were made earlier than in *A* and it was found that the cultures kept at the lower temperatures, developed much more slowly than at the higher ones, such as laboratory temperature. Series *B*1 and *B*2 were still in rather poor condition on the seventh day while *B*4 was in good condition and a few cysts were present.

As noted above, a very few cysts appeared in five of the cultures of *A*1. This was not true for *B*1 as no encystment whatever occurred. The experiment was discontinued on the fifty-first day and seven cultures of series *B*1 had completely died out, the remaining five were in fair condition. These five were permitted to remain in the laboratory and in five days they had completely died out without any encystment.

We see that, as a whole, no encystment occurs at extremely low or high temperatures. Behavior at high temperatures differs from that at low in that, in going up the scale, encystment is not prevented until a point is reached which will kill the flagellates in a few days, while at low temperatures they develop more slowly and make very long lived and good cultures.

How are these results to be interpreted? At low temperatures one would expect the rate of metabolism to be lower and the time before encystment to be longer, but when the cultures died out without encystment a new light was thrown on the problem. If encystment were a response to adversely low temperatures we would expect it to occur there first. On the other hand, if encystment were a response to adversely high temperature we would think that the two days which the cultures lasted at 35° C. would have been sufficient for the organisms to encyst rather than die. It is also noteworthy that the six cultures at 30° did not encyst until after their controls. It seems entirely justifiable to conclude that extremely low or high temperatures prevent encystment and that medium temperatures are not only conducive to rapid growth and division but also to encystment.

Hydrogen-ion Concentration.—It was noted in the above account that room temperature was the best one employed for the growth and division and encystment of *Polytomella citri*. Consequently, all of the remaining experiments were carried out at room temperature.

The procedure for the work on hydrogen-ion concentration has largely been given. Unaltered hay infusion (P_h 5.4) was used for the controls and, of course, these were not altered for the duration of the experiment. For the remaining cultures the P_h was restored to that at which the cultures were started on the third, ninth, and sixteenth days. Forty-eight cultures were seeded and the hydrogen-ion concentration was changed to the following P_h values (six cultures in each series): 3.5 (series *C1*), 4.5 (series *C2*), 5.4 (series *C3*), 6.2 (series *C4*), 7.5 (series *C5*), and 8.5 (series *C6*). Twelve cultures were kept as controls (series *C7*).

By the third day the cultures of series *C1* were in fair condition and so they remained for the first week. After that the numbers decreased to such an extent that all could truly be classed as poor cultures. On the twenty-second day they were still poor and no cysts had been formed. Series *C2*, *C3*, *C4*, and *C5*, were practically identical throughout the first twenty-two days. This correspondence was so great that it is unnecessary to give separate results for each, which would be merely repetition. They were in good condition on the third day and excellent on the fifth. A very few cysts appeared on the fifth day. On the twenty-second day, the cultures were still in good condition and some cysts were present. Series *C6* remained rather poor throughout the experiment and only a very few cysts were found on the twenty-second day.

The controls (*C7*) behaved quite differently from the other cultures. On the fifth day all twelve cultures of *C7* were in excellent condition and some cysts were present. By the eleventh day many cysts were found, while in the other series this condition was never reached. The cultures were only fair on the twenty-second day and very many cysts were present.

We now see that the cultures did excellently at any P_h from 4.5 to 7.5 and that encystment occurred with equal facility at any point within that range. However, in no experimental case was encystment as great as in the controls.

This experiment has been followed only to the twenty-second day, but observation was not discontinued at that time. They were examined on several occasions, but it will suffice to give only one of these, that made on the thirty-fourth day. All cultures were rather poor, so far as active forms are concerned and many cysts were present, though not as many as were found in the controls. This data, together with some which will follow, indicates that it was not the actual hydrogen-ion concentration which prevented the experimental cultures from equaling the controls in rapidity of encystment, but the sudden changing of the medium when the P_h was altered.

These results are quite contradictory to those of Koffman, and are more in harmony with Brand's conclusion that chemical influences are of little importance in producing encystment.

By-Products of Metabolism and Food Supply.

These two are taken up together partly because similar methods were used in dealing with them and partly because both factors entered into some of the experiments.

An attempt was first made to determine the effect of removing the by-products of metabolism by filtering the cultures through a porcelain filter and replacing the medium with fresh hay infusion. Twenty-four cultures were seeded and six of these had their culture medium replaced with fresh hay infusion every day (series *D1*) six others every second day (series *D2*), six others every third day (series *D3*) and the remaining six were kept as controls (series *D4*). The medium was not replaced in any of the cultures after the tenth day.

The results of this work are very startling. Whereas in all previous experiments the controls behaved beautifully, encysting in about a week, the controls in this case did not encyst until the tenth day and then very few cysts appeared, most of the active forms dying rather than encysting. Although all the cultures of *D*4 (controls) were in excellent condition on the eighth day they had entirely died out by the fifteenth day and few cysts were present. The cultures of series *D*2 and *D*3 did not die out as soon as those of *D*4 nor did they encyst as quickly. On the twenty-second day of the experiment they had reached the same condition in which *D*4 was found on the fifteenth. By the thirtieth day all active forms had disappeared from the cultures of *D*1 and slight encystment had occurred.

Not knowing what to make of this behavior, twenty-four more cultures were seeded and the experiment repeated. These cultures were divided into four series *E*1, *E*2, *E*3, and *E*4, each series corresponding to the similarly numbered series of *D*. This yielded identically the same results as *D* with the exception that *E*1 and *E*2 died out without any encystment whatever.

It is well to state here that *D* was seeded from a culture of *C*1 shortly before it completely died out and *E* was seeded from a culture of *C*2 after it had reached a very poor condition. No cysts were transferred, only active individuals. Series *C* had likewise been seeded from *A*3. In an attempt to find the explanation for this loss of ability to encyst on the part of series *D* and *E*, some cysts were taken from *C*6, placed in fresh hay infusion and permitted to excyst. On the next day eighteen cultures were seeded from these newly excysted ones and two days later it was found that every one of the eighteen contained many cysts. As a check on this, cysts were taken from *C*7, washed in seventy per cent. alcohol, and permitted to excyst in fresh hay infusion. From these, thirty-six cultures were seeded and on the second day they were found to have repeated the activity of the eighteen, being largely encysted. In addition, cysts were taken from *E*3, washed in alcohol, excysted, and twelve cultures seeded from them. At the end of two days many cysts were found in all of the cultures.

By putting the last two paragraphs together, one gets a strong indication that the longer *Polytomella citri* is removed from encystment, the less is their tendency to encyst.

To carry this further, series *F* was started. *F*1 (six cultures) was seeded from *A* 1 on the sixtieth day after that series was seeded; *F*2 (six cultures) was seeded from *E*2 on the eighteenth day; *F*3 (six cultures) was seeded from active ones that were ten days removed from newly encysted individuals; and *F*4 (eighteen cultures) was seeded from active ones excysted only five days previously.

On the third day all cultures were excellent, with the exception of *F*1, which were in fair condition. No cysts had appeared in the first three series, but some were present in the cultures of *F*4. By the seventh day *F*1 and *F*2 were in excellent condition, with no cysts while the cultures of *F*3 were in good condition with some cysts and *F*4 was only fair with many cysts. Examination on the nineteenth day revealed the fact that the cultures of all four series were very poor. A very few cysts had appeared in several of the cultures of *F*1; there were none at all in *F*2 while *F*3 and *F*4 contained many cysts.

It is now clearly seen that the longer encystment is prevented, whether by low temperature (*F*1) or by long continued transfer (*F*2), the less is the tendency to encyst, and if this procedure be sufficiently extended the ability to encyst is entirely lost. The morphological basis for this is given in the next section.

It was determined to repeat the work of *D* and *E*, using newly excysted forms. Accordingly twelve cultures were seeded and six of these had their medium replaced every day, the others were kept as controls. In two days the controls were quite generally encysted while those filtered every day showed no cysts until the third day.

Some cultures of the types found in *F*2 and *F*4 were filtered and the medium replaced with fluid taken from cultures that had entirely encysted. In the former no encystment whatever occurred, while in the latter it was slowed up, usually for about one day, as compared to the controls. When first placed in such a medium, we were surprised to find that division was apparently stimulated, as examination under the microscope would reveal a great many late division stages, which are the only ones that can be detected in living material. Although encystment was slightly deferred in the case of those of type *F*4, the cultures themselves in all instances ceased to show any active forms before they disappeared in the controls. This was probably due to the lack of food.

Now let us inquire why *E*1 and *E*2 did not encyst at all. Was it because of the removal of the by-products of metabolism? The evidence is very much against such a conclusion. If that were true, we should have expected the cultures of the last mentioned experiment to have encysted before the controls, since in that case metabolic by-products were probably increased. Also, in the case of the cultures seeded with newly excysted individuals in which the medium was replaced with fresh hay infusion every day, we should have expected encystment to have been postponed more than it was, if metabolic by-products were an important factor for encystment. Again in series *E*1 and *E*2 active forms remained in the cultures for ten days after filtering was stopped. This, we think, would have been sufficient time for the necessary by-products to accumulate, yet no encystment occurred. The arguments just recounted seem to entirely justify the conclusion that the filtering of a culture and replacing its medium with fresh hay infusion defers encystment, but this effect is produced by the stimulus to growth and division given the organisms by changing the medium suddenly, and is not due to the removal of an adverse environmental factor created by the presence of metabolic by-products. This is quite comparable to seeding a new culture.

The reader will doubtless have thought of the fact that an ever plentiful supply of food was present during the early course of *D*1, *D*2, *D*3, *E*1, *E*2, and *E*3. However, the same arguments that applied to metabolic by-products will apply here and we can likewise conclude that food deficiency is not the cause of encystment. This was carried further by filtering thriving cultures and replacing their medium with distilled water, 25 per cent. hay infusion and 75 per cent. distilled water, etc. to pure hay infusion, a change of 25 per cent. in each step. In the cultures that received pure distilled water no encystment took place and the cultures died out in two days. In the others, however, neither fission nor encystment seemed to be materially affected.

A few experiments were carried out which may have involved oxygen deficiency. Thriving cultures of Polytomella were sealed in glass tubes, very little air space being left between the top of the medium and the sealed end. Since no photosynthetic organisms were present, it would seem as though the dissolved oxygen of the medium should have decreased. At the end of one week the cultures had died out and very few cysts had been formed.

MORPHOLOGICAL CHANGES ACCOMPANYING THE EXPERIMENTS.

When a hay infusion culture is seeded with *Polytomella citri* taken from a stock culture (one containing uncooked hay in distilled water) the organisms will multiply rapidly for a few days, during which time staining with iodine will indicate the presence of relatively little starch (Fig. 1*a*). At the end of from two to five days it will be noticed that the starch increases to such an extent that the entire cell seems filled with it (Fig. 1*b*). It is at this time that encystment occurs. Polytomella never encysts except when the cell-body is filled with starch, and, on the other hand, such a culture has never been found to be unaccompanied by cysts.

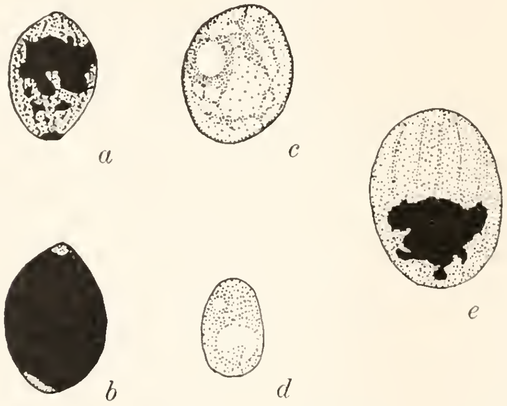

FIG. 1. Diagrammatic figures traced from photomicrographs of Polytomellas stained with iodine. Solid black indicates starch. Equal magnification. (See text for further explanation.)

As stated above, all cultures used in this work were derived from a single stock, and later cultures were seeded with active ones taken from previous experiments.

It was not only found that when *Polytomella* is so transferred from culture to culture for some time their tendency to encyst becomes practically negligible, but also that marked morphological changes occur. When encystment is thus prevented for some time the organism will frequently show considerable decrease in size, almost invariably the cytoplasm will become coarsely vacuolated, and the starch entirely disappear. Fig. 1*c* was taken from series *E*4 on the fourteenth day after seeding; Fig. 1*d* from series *A*1 on the sixtieth day. In series *D*1, *D*2, *D*3, *E*1, *E*2, and *E*3, it was noticed that all contained little starch. In living mounts the cell-body was quite clear and the size above normal. Fig. 1*e* was taken from *E*2 on the eighteenth day. The abnormally large size was lost as soon as the numbers began to decline. Eventually all of these cultures showed only a few degenerate, vacuolated individuals.

It was thought desirable to determine whether or not these degenerate forms could give rise to healthy cultures. Accordingly, series *F* was started. The history of these cultures has already been given, with the exception of the data found in Fig. 2. Fig. 2*a* was made from a typical individual taken from

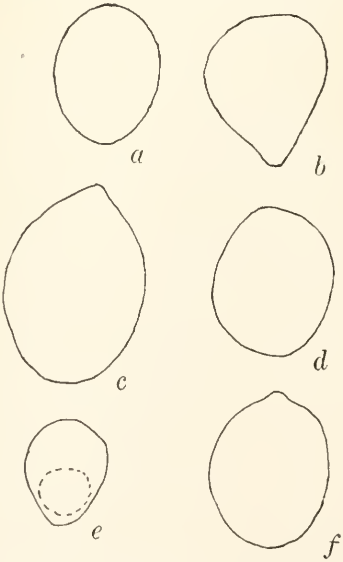

FIG. 2. Camera-lucida outlines showing variations in size. Figure e vacuolated. (See text for explanation.) Magnification 1825 ×.

the culture which furnished the starting point for *F4*; Fig. *2b* was taken from *F4* two days later. Fig. *2c* was taken from *E2* on the day that *F2* was seeded; *2d* was taken from *F2* two days later. Fig. *2e* was taken from *A1* on the day that *F1* was seeded; Fig. *2f* from *F1* on the second day. A glance at these figures will show that transference of these degenerate forms to a new and favorable medium results in a return towards normality, with the exception of the formation of starch, as *F1* and *F2* formed little of that material.

DISCUSSION.

The Causes of Encystment.—In the preceding section attention was called to the fact that *Polytomella citri never encysts except when the cell body contains abundant starch.* Since this is so it seems as though the search for the external factors for encystment becomes resolved into a search for the factors which encourage the formation and storage of starch. Adverse conditions of the environing medium certainly would not be conducive to the formation of this material, at least the above experiments so indicate, and, consequently, we need not be surprised at the conclusion drawn from the present work, namely, *Polytomella citri* encysts only under such conditions as are favorable for growth and reproduction, and adverse conditions lead to death of the culture rather than encystment. This very thing, however, may prolong the life of a culture, so far as active forms are concerned.

The presence of abundant starch in *Polytomella* as a prerequisite for encystment is quite in harmony with the observations of Hogue on *Amœba limax*, Carter on *Amœba proteus*, Kofoid and Swezy on marine dinoflagellates, Stolte on *Blepharisma*, Mast and Ibara on *Didinium*, and Hall on *Oxyrrhis marina*. In fact, most of the recent work indicates that abundant food is much more conducive to encystment than is starvation, which was formerly supposed to be one of the principal causes of encystment, and is still so given in text-books.

In the introduction a quotation was given from Koffman (1924). From this it is seen that altering the P_h to a lethal point either resulted in death or encystment, generally death.

From this he concludes that hydrogen-ion concentration is probably one of the most important factors in producing encystment. It seems to us that he would have been justified in concluding that adverse hydrogen-ion concentration might slightly speed up the process, if the internal condition of the protozoan disposed it towards encystment. It is just such conclusions as Koffman's that have led to an over-emphasis of the importance of adverse environmental factors in producing encystment and have led to some authors speaking of the phenomenon as a "passive adaptation to adverse environment." Although that may be true in a few instances, a critical review of the literature certainly does not indicate that it is the general rule.

The Nature of Encystment.—In a former paper Kater has shown that extensive changes occur during the encysted stage of *Polytomella citri.* This does not particularly involve the nucleus, the budding off of the new centriole and basal granule from the karyosome being the only possible regenerative change that was found. The most striking changes take place in the cytoplasm. The starch rapidly disappears when this resting phase is entered, and, coincident with its disappearance, the metachromatic granules are formed in great abundance. These are absorbed before excystment occurs.

Before attempting a discussion of the meaning of encystment in this flagellate, we must decide why prevention of encystment for several months results in loss of the tendency to store starch, and, consequently, to encyst. On a priori grounds we can say that it is either due to selection, direct effect of the environment, or a combination of the two. Although we may not be able to answer this conclusively, the present evidence indicates that selection does not enter into the matter. If it were due to selection of an encysting strain we should not expect cysts taken from *E*3 and *C*7 to give rise to cultures that would encyst in exactly the same time, two days, yet such is the case. *C*7 encysted much more quickly than did *E*3 and, if there were anything in the strain, the offspring should behave likewise. In the second place, *F*1 showed only a very slight tendency to encyst, mostly dying. Since these cultures were seeded from

*A*1 on the sixtieth day we must suppose, if it is a matter of selection, that the non-encysting variations are more viable than the encysting. This does not seem reasonable, because if it were so we would hardly expect any existing *Polytomella* to exhibit the phenomenon.

No attempt has been made to accurately measure the division rate in a non-encysting strain, such as *F*2, as compared to an encysting, *F*4. Mass observations lead us to believe that even when *Polytomella* has reached a condition of morphological degeneracy and may have entirely lost the power to encyst when they are transferred to a fresh and suitable medium, they are stimulated to a perfectly normal period of growth and division, with the exception that it does not end in encystment.

The morphological degeneration observed in the cultures *A*1, *B*1, *D*, *E*, etc., is very similar to that noted by Maupas in *Stylonychia* when conjugation was prevented and by Calkins in *Uroleptus*. We will not enter into the propriety of terming this senescence, and encystment as a rejuvenating process.

SUMMARY.

1. *Polytomella citri* encysts only when the cell-body contains considerable starch.

2. Encystment is not due to any perceptibly adverse environmental factors.

3. Optimum conditions for growth and reproduction are concomitant with maximum encystment.

4. Prevention of encystment, either by continuous transfer or by low temperature, if carried to sufficient extent, will result in morphological degeneracy and loss of the tendency to store starch and to encyst.

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SEQUENCE OF CORRESPONDING THIRD-CHROMOSOME GENES IN *DROSOPHILA MELANOGASTER* AND *D. SIMULANS.*

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It has been shown (Sturtevant, 1921*a*, *b*) that *Drosophila melanogaster* and *D. simulans* may be crossed, and accordingly it is possible to test suspected corresponding genes in the two species. There is, for example, a recessive vermilion-like eye-color (known as scarlet) in each species. If scarlet of either species is crossed to the wild type (or to any other eye-color) of the other species, the resulting hybrids have the wild-type or "red" eye-color. Therefore each species carries the dominant wild-type allelomorph of the scarlet mutant of the other species. But if scarlet *melanogaster* is crossed to scarlet *simulans*, the resulting hybrids have scarlet eyes. Therefore the two scarlets must represent modifications of the same wild-type gene, or at least of wild-type genes that have like effects on development. Such genes as the two scarlets are described in this laboratory by the term "corresponding," rather than (as formerly) "allelomorphic"—since it seems desirable to reserve the latter term for genes that regularly separate at maturation. In the present case the fact that all the hybrids are sterile prevents any test of the segregation.

A study of thirteen corresponding genes in the X-chromosomes of *melanogaster* and *simulans* (Sturtevant 1921*a*, and unpublished data) shows that the sequence of the loci concerned is the same in the two species. But it was shown by Sturtevant (1921*c*) that the sequence is not identical in the third chromosomes. If the three corresponding genes scarlet (st), peach (p), delta (Δ, formerly called "deltoid" in *simulans*) are mapped, the sequence is found to be stpΔ in *melanogaster*, stΔp in *simulans*.

The present paper is a report on three additional pairs of corresponding genes in this chromosome, which make it possible to compare the maps in more detail. The three loci concerned, already well known in *melanogaster*, are sepia, hairless, and claret. In the cases of sepia and claret the mutant types of *simulans* are closely similar in appearance to the types of the same names in *melanogaster*, and it was a simple matter to obtain sepia hybrids and claret hybrids by crossing the types concerned. The evidence that these are corresponding genes is thus of the same nature as in the cases of scarlet, peach, and the sex-linked genes previously reported (Sturtevant, 1921*a*, *b*).

Hairless is, however, dominant in both species, so that the same difficulty was encountered here as in the case of delta—the hybrids show the mutant character even when it is introduced from only one parent. But in each species hairless, like delta, is lethal when homozygous; accordingly this lethal effect was used to test the correspondence of the hairless genes. Since the earlier tests of delta were not as clear-cut as might be desired, the new tests were carried out with flies containing both genes, and served to show that both loci correspond.

D. melanogaster females that were Δ/H—i.e., carried delta in one third chromosome and hairless in the other one—were crossed to *simulans* males that were also Δ/H. There resulted 169 hybrid offspring: 164 delta hairless, 4 delta, 1 hairless, 0 wild-type. If the delta genes do not correspond, so that Δ/Δ is viable, the cross should give half as many delta flies as delta hairless; if the hairless genes do not correspond, the hairless class should also be half as large as the delta hairless class. The few delta and hairless offspring produced are evidently due to crossing over in the mothers, 1.5 per cent. of whose eggs are expected to carry neither Δ nor H. The result was checked by crossing *melanogaster* females that were $\Delta H/+$ by *simulans* males that were Δ/H. From this cross, all the ΔH eggs should give inviable zygotes, and equal numbers of delta and hairless offspring should result. There were obtained the following hybrids: 1 delta hairless, 225 delta, 226 hairless, 0 wild-type. The one delta hairless remains unexplained, for in this case the mothers carried C_{III}, which should have prevented all crossing over between Δ and H. This individual probably represents a new mutation, though it is possible that it was really homozygous for Δ or for H, since the somatic effects produced by these genes are in many respects on the same characters but in opposite directions, so that heterozygosis for one of them might conceivably tend to counteract the lethal effect of the other one. Since all the *melanogaster-simulans* hybrids are sterile, it was not possible to test this exceptional individual. In any case, this one specimen cannot vitiate the conclusion that both delta and hairless are corresponding genes in the two species.

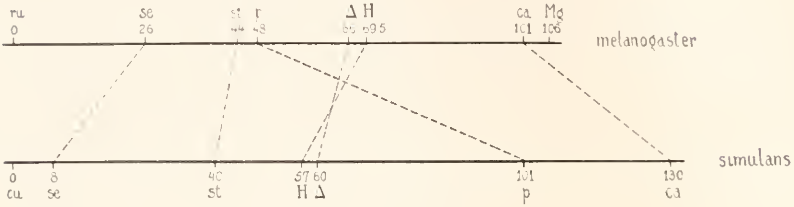

FIG. 1.

Fig. 1 shows the linkage relations of the six corresponding genes in the two species, together with the left-most and right-most known loci of each species.

The *melanogaster* map is based on the values given by Bridges and Morgan (1923). The simulans map is based on unpublished data, including counts from crosses involving several genes not here recorded. The intervals in this map are not yet accurately determined, because of a lack of favorable mutations; but they are more likely to be lengthened than to be shortened by further data. -The sequence of the corresponding genes in simulans is clearly established, as the following data will show. Claret cannot be distinguished somatically from peach, so its position

TABLE I.

$$\frac{+\ \mathrm{st}\ +}{\mathrm{se}\ +\ \Delta}\ ♀ \times \mathrm{se\ st}\ +\ ♂.$$

Non-Crossovers.	Single Crossovers.		Double Crossovers.	Total.
0.	1.	2.	1, 2.	
st seΔ	Δ sest	stΔ se	+ sestΔ	
121 142	70 44	43 79	21 8	528

has been determined with respect to minute, which is very close to it.

TABLE II.

$$\frac{+\ \mathrm{H}\Delta\ +}{\mathrm{st}\ +\ +\ \mathrm{p}}\ ♀ \times \mathrm{st}\ +\ +\ \mathrm{p}♂.$$

0.		1.		2.		3.		1, 2.		1, 3.		2, 3.		1, 2, 3.		T.
HΔ	stp	p	stHΔ	Hp	stΔ	HΔp	st	Δ	stHp	+	stHΔp	H	stΔp	Δp	stH	
320	330	69	75	7	11	181	181	3	0	41	29	3	1	0	1	1,252

Minute (which is dominant) sometimes overlaps wild-type in appearance; accordingly only the minute offspring have been recorded in Table III. In the experiment reported in Table IV.

TABLE III.

$$\frac{+\ +\ +\ +}{\mathrm{st}\Delta\mathrm{pM}}\ ♀ \times \mathrm{st}\ +\ \mathrm{p}\ +\ ♂.$$

0.	1.	2.	3.	1, 2.	1, 3.	2, 3.	1, 2, 3.	T.
stΔpM	ΔpM	pM	M	stpM	stM	stΔM	ΔM	
126	48	77	51	22	25	28	4	381

TABLE IV.

$$\frac{+\ \Delta\ +\ \mathrm{M}}{\mathrm{st}\ +\ \mathrm{ca}\ +}\ ♀ \times \mathrm{st}\ +\ \mathrm{ca}\ +\ ♂.$$

0.		1.		2.		3.		1, 2.		T.
ΔM	stca	stΔM	ca	stM	Δca	stcaM	Δ	M	stΔca	
39	19	4	2	24	18	0	2	8	3	119

this overlapping seemed to be slight, so all the offspring are recorded. It is possible, however, that the two M-ca crossovers may be due to such overlapping. More extensive counts from M/ca females, in experiments in which no other loci were studied, have not given any crossing over. There is an unusually low scarlet delta crossover value shown in Table IV., but the count is so small that no special significance is to be attached to this fact.

There can now be no doubt that one half or more of the right limb of the third chromosome (in terms of map distance) has been inverted in one or the other species—since there is no clue as to which is the original sequence. The left-hand limit of the inverted section lies between scarlet and peach of *melanogaster*, which is presumably very close to the spindle-fiber attachment, and is certainly in a region where the mutant genes are mapped very close together and where coincidence of crossing over is very high (Bridges and Morgan, 1923). It will be interesting, as more mutant genes are studied in *simulans*, to see how this same material behaves when it lies further from the middle of the chromosome.

In the earlier account (Sturtevant, 1921*c*) it was suggested that the rearrangement of parts demonstrated for the *melanogaster* and *simulans* third chromosomes might be explained as resulting either from a duplication or from an inversion of a section. The new data here reported speak strongly for the inversion interpretation.

One might surmise that this inversion of a piece of a chromosome is the fundamental factor in the specific difference between *melanogaster* and *simulans* and the sterility of their hybrids. But it has been shown (Sturtevant, 1920) that at least some of the factors concerned in the inviability of certain classes of the hybrids are in the X chromosomes, where there has certainly been no such inversion.

This case shows that identity of sequence is not a necessary condition for correspondence of genes in different species. It also suggests that we need not be surprised should such an inversion be found within a species.

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SYSTEMIC AND SEX DETERMINANTS OF BONE GROWTH (*MUS NORVEGICUS ALBINUS*).

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Studies of the chemical differentiation of bone during growth ('25*a*, *b*, *c*) and a comparison of bone growth in length with that in weight ('25*d*) indicated, as might be expected, that systemic and sex factors are participants in the determination of the mode and rate of development of this type of structure. It is therefore pertinent to attempt an evaluation of the influence of these factors in order that knowledge in this direction be extended and rendered more precise. Such has been done for the chemical differentiation and has been reported in the papers cited. This paper deals with their evaluation in the growth of the bones in weight and length.

The raw data were obtained from the same source as that which served for the previous studies, and hence are directly related thereto. The material analyzed consisted of the lengths and weights of the humerus and femur of male and female albino rats 23, 30, 50, 65, 75, 100 and 150 days of age, together with the respective body lengths and weights. The mean values and their probable errors are given in Table I.

The distribution of the material has been given ('25*a*) and need not be repeated. The animals were healthy. They came from the Experimental Colony stock of the Wistar Institute, had a common inheritance, and were raised under like conditions of diet and environment as described by Greenman and Duhring ('23).

Data on the osseous system of the albino rat on age have been reported by Jackson and Lowery ('12) and Donaldson and Conrow ('19). Their records do not lend themselves to an analysis, such as the present, because of the lack of systemic or sex separation.

TABLE I.

THE MEAN BONE AND BODY LENGTH AND WEIGHT AT THE STATED AGES.

	Length.			Weight.		
	Humerus.	Femur.	Body.	Humerus.	Femur.	Body.
Day.	Mm.	Mm.	Mm.	G.	G.	G.
			Male.			
23	13.4 ± 0.06	14.8 ± 0.09	95.3 ± 0.74	0.0557 ± 0.0020	0.0841 ± 0.0035	27.3 ± 0.62
30	15.7 ± 0.08	18.4 ± 0.11	116.1 ± 0.83	0.0786 ± 0.0021	0.1373 ± 0.0041	41.4 ± 0.81
50	18.3 ± 0.08	22.7 ± 0.13	143.2 ± 0.59	0.1018 ± 0.0016	0.2069 ± 0.0027	74.5 ± 1.80
65	21.2 ± 0.15	27.0 ± 0.24	169.3 ± 1.15	0.1488 ± 0.0035	0.3255 ± 0.0085	120.8 ± 2.05
75	21.9 ± 0.10	27.9 ± 0.22	174.8 ± 1.17	0.1591 ± 0.0028	0.3407 ± 0.0060	133.2 ± 3.24
100	22.9 ± 0.27	29.7 ± 0.33	182.5 ± 1.60	0.1847 ± 0.0055	0.4050 ± 0.0131	162.3 ± 4.60
150	25.9 ± 0.27	33.9 ± 0.33	205.8 ± 1.60	0.2556 ± 0.0041	0.5811 ± 0.0198	244.3 ± 6.90
			Female.			
23	14.0 ± 0.07	15.5 ± 0.10	98.9 ± 0.84	0.0573 ± 0.0017	0.0885 ± 0.0034	28.5 ± 0.70
30	15.3 ± 0.06	17.9 ± 0.09	110.8 ± 0.63	0.0725 ± 0.0015	0.1258 ± 0.0031	38.7 ± 0.66
50	18.2 ± 0.10	22.6 ± 0.16	141.8 ± 1.04	0.1008 ± 0.0019	0.2011 ± 0.0042	74.2 ± 0.79
65	20.5 ± 0.13	26.1 ± 0.17	161.8 ± 0.90	0.1333 ± 0.0023	0.2866 ± 0.0042	105.0 ± 1.55
75	21.2 ± 0.15	27.0 ± 0.21	166.8 ± 1.34	0.1417 ± 0.0037	0.3080 ± 0.0077	115.8 ± 2.56
100	22.5 ± 0.15	29.0 ± 0.23	177.1 ± 0.90	0.1732 ± 0.0031	0.3818 ± 0.0090	137.6 ± 2.20
150	24.0 ± 0.13	31.0 ± 0.30	188.4 ± 1.30	0.2033 ± 0.0050	0.4502 ± 0.0119	174.7 ± 4.40

Since growth is a process it should be studied as such and expressed in terms of rate. This point of view governs the present inquiry.

From Table I. it is seen that the femur is heavier and longer than the humerus at all ages in both sexes. This is a consequence of the fact that the rate of growth in weight and length (grams

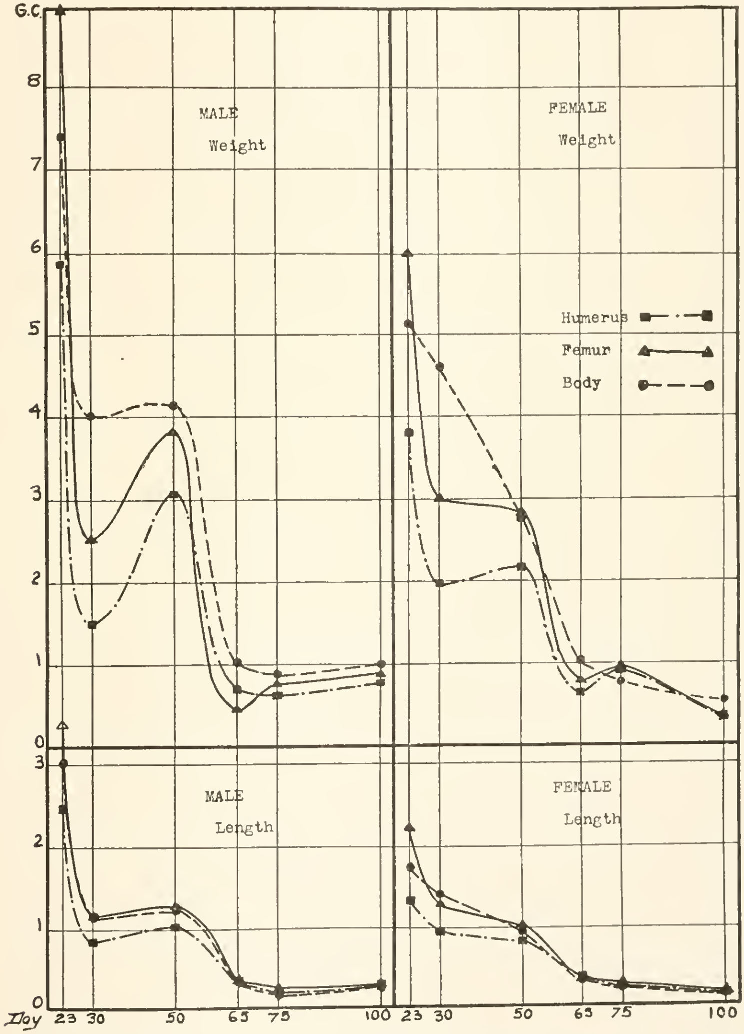

CHART I. The growth capacity in weight and length on age of the humerus, femur and body of the male and female albino rats.

and millimeters per day) is greater in the former than in the latter.

Chart 1 depicts the values of the growth capacity (grams per 100 grams and millimeters per 100 millimeters per day) of the bones and body weight and length at the stated ages.

It shows that the growth capacity of the femur in weight and length is generally greater than that of the humerus in both sexes up to 65 days of age, and that thereafter the differences are inconsiderable. As a result of this superiority the femur becomes increasingly larger and heavier than the humerus up to the age of 65 days, at which time systemic divagation is brought practically to an end by the approximation to a common level of the growth capacities of the two bones. At 65 days of age, then, the systemic difference has reached the level characteristic of the adult animal. The cessation of deviation at this time indicates that the period between 23 and 65 days of age is the period of systemic development of these bones just as it is the period of their differential chemical development, and that the culmination of puberty at 65 days acts as an equilibrating agent with the one as with the other.

In both sexes the growth capacity of the humerus in length is quantitatively more nearly like that of the femur than is the growth capacity in weight. As a result the increase in difference in length between the two bones is less in degree than the increase in weight difference. Hence the systemic difference in serially homologous bones in the adult as in the immature animal is less a difference in length than a difference in weight.

The ratio of the growth capacity of the humerus to that of the femur in both weight and length is generally of the same order of magnitude in both sexes at all ages. But the chart shows that there are sex differences in the course of the changes in growth capacity with age during the period of differential development. However, the course tends to be systemically characteristic even though sex-specific in type. From this it can be concluded that these systemic relations are practically sex-indifferent. They are however, probably species specific.

It is clear from the chart that there occurs a decrease in growth capacity in weight and length of bones of both sexes on age.

This decrease is not uniform but largely takes place in two stages at which the reduction in growth capacity is markedly abrupt. These occur at 30 and at 65 days of age. The latter brings the growth capacity to the level which is approximately the same for all bones and for both phases of growth, which is the level maintained thereafter, and which is the level characteristic of adult growth.

The reduction at 30 days is attributable to the adjustment to weaning, that at 65 days to the adjustment to puberty. These phases of development have been discussed elsewhere ('25*a*).

There is no apparent systemic difference in degree of response to weaning. With respect to the pubertal reduction, however, a consistent difference is exhibited, in that the degree of cut in growth capacity is greater for the femur than for the humerus in both sexes.

This systemic difference can be considered as a particular expression of the general tendency of the growing organism to reach that state of uniform level of growth capacity of all of its parts, wherefrom those bodily proportions are maintained which characterize the adult of the species. (The basis of this idea has been sketched in an earlier publication ('23*a*): the full discussion will be developed in a later analysis.) Assuming the correctness of the hypothesis, it is obvious that since the growth capacity of the femur during the preceding period of active differential development is greater than that of the humerus, adequate systemic approximation in growth capacity, which is brought about in the case of these two serially homologous components of the osseous system by the culmination of puberty, is only arrived at through a differential systemic reduction in growth capacity such as is shown here.

The chart shows that in both sexes the growth capacity of the femur in weight and length is more nearly like that of the body in degree than is that of the humerus. In other words the growth of the femur follows that of the body more closely than does the growth of the humerus. The precise significance of this systemic difference is obscure. It may be an expression of a response to a need for supporting the greater part of the body weight included in the posterior abdomen. In this connection it might be noted that the growth capacity of the femur in length generally tends to be slightly greater than that of the body, while that of the humerus is less.

Attention should be directed to the fact that in both sexes there occurs at 65 days of age an approximation of the growth capacity values in body weight and length to a common level, which is quite the same in character as that exhibited by the bones and their constituents, and which is similarly interpretable.

The foregoing analysis demonstrates that the systemic factors participating in the determination of bone growth are largely productive of differences in the degree rather than in the course of development.

Turning now to the inter-sex comparison it is seen from Table 1 that both bones are consistently slightly heavier and longer in the male than in the female save at 23 days of age. The direction of sex difference in the bones, however, is at all ages positively correlated with the direction of sex difference in body size.

A study of Chart 1 reveals the fact there is no consistent direction of sex difference in growth capacity of the bones on age. At 30 and at 75 days of age the growth capacity values are greater in the female than in the male; at 23, 50, and 100 days the reverse is the case, while at 65 days the differences are inconsiderable. It is to be noted, however, that the differences in growth capacity values induced by sex-specific factors affect the bone as a whole and not differentially, in so far as the direction of difference is concerned.

Notwithstanding the inconsistency of sex difference on age, it is a fact that the sex difference in growth capacity in length of both bones is generally less than the sex difference in weight. As a result the degree of sex difference of the bones in length is less than that in weight at all ages.

The general principle is derived from this and the similar relation exhibited in systemic development, that the differentiation of the long bones during growth is due more to the process of growth by increments in weight than to those concerned in growth by increments in length.

This is substantiated by the fact that the increase in sex difference which occurs in both bones, is greater in the weight than in the length relation.

In the analysis of the systemic determinant of bone growth it was shown that a marked reduction in growth capacity takes place at 30 and 65 days of age, and that there is a systemic difference in the degree of reduction at puberty, but not at weaning. It is otherwise in the sex relations, for at both stages of development a sex-difference in degree of reduction in growth capacity obtains. This reduction is uniformly greater in the bones of the male.

The sex differences in the degree of pubertal response are interpretable on the same basis as that given for the systemic differences. The interpretation of the sex-difference in degree of response to the weaning adjustment is, however, another matter. It is probable that a sex-specific factor for ossification is the major influence at this period. This conclusion is based on the fact that an acceleration of ossification or growth capacity for ash, occurs in both bones in the female at 30 days of age, while in the male the usual reduction is exhibited. Since the degree of reduction in the growth capacity of the bone as a whole is dependent upon the degree of reduction of its constituents, it is obvious that a change such as described must produce the numerical dissimilarity exhibited, other relations being approximately the same.

A sex difference in type as well as in degree of development is exhibited in these bones. From the chart it is seen that an acceleration of growth capacity in weight and length occurs in the male but not in the female at 50 days of age. This indicates that a stimulus is active at this time in the male which is not expressed in the female. It is largely a stimulation of the processes of ossification which is sex-specific in character. A like tendency is exhibited in the bones of the female at 75 days of age. The implications of these differences have been discussed elsewhere ('25*a*). All that is necessary to point out here is that the sex factors participating in the determination of bone growth are productive of differences in the course as well as in the degree of differential development, and are thus unlike the systemic factors which apparently are solely productive of differences in degree or rate of growth.

An inspection of the chart shows the presence of a tendency for the growth capacity of the bones of the male in weight and length to be more typical of that of the body than is that of the bones of the female during the period of active differential development.

The course of development of the bones in weight is sex-specifically different from that of the body. This major difference occurs at the same time in each sex (*e.g.*, during the growth period from 30 to 50 days of age). In the male the growth capacity of the bones increases, while that of the body remains practically constant: in the female the growth capacity of the bones remains practically constant, while that of the body is markedly decreased. An explanation of these differences is not at hand. It is sufficient for the present purpose to point out that the determination of the course of growth of these bones in relation to that of the body is factored by sex.

From the foregoing it is evident that systemic correlation is closer than sex correlation, in both rate and type of development, particularly during the period of active differentiation. This indicates that the sex-specific factors of influence are superimposed upon the systemic specific determinants of bone growth. These sex-specific factors are probably as much if not more attributable to immediate incretory influences emanating from the gonads, than to the more remote and generalized determinants of genetic origin. This belief is sustained by the fact that the occurrence of these differences is correlatable in time of appearance with the exhibition of certain sex-specific stages in gonadal development. This has been discussed in detail in the report on the chemical differentiation of the bones ('25*a*).

Certain other derivatives are of biological interest.

As a result of sex differences in the relative rates of growth, the length of the bones tends to be greater in proportion to the body length in the female than in the male, at all ages. This indicates that the appendicular skeleton of the female when compared with the axial is longer than that of the male.

A similar superiority of the humerus and femur of the female in weight with respect to body weight is exhibited from 65 days of age on. It is possible that the skeletal system of the sexually mature albino rat is a greater proportion of the body as a whole in the female than in the male.

The weight of the femur per unit bone length is greater than that of the humerus at all ages. The weight of both bones per unit length is greater in the male than in the female in all cases save the femur at 23 days of age. The systemic difference is consistently greater than the sex difference. These differences are structural differences, and indicate, as would be expected, that there is a wider divergency in the nature of the genetic factors concerned in structural differentiation of serially homologous bones, than there is between the sex-differential factors concerned in the development of isotropic bones.

In the comparison of bone growth in length with bone growth in weight ('25*d*) it was shown that there is a high degree of positive linear correlation in both weight and length in both sexes, with body weight and length respectively, and that systemic correlation in both length and weight was also positive, linear and high. The values recorded represented the inter-structural association values with the age factor unstabilized.

TABLE II.

FIRST ORDER CORRELATION COEFFICIENTS.

	Male.	*Female.*
Humerus weight and femur weight (body weight constant)........	0.844 ± 0.0042	0.727 ± 0.0030
Humerus length and femur length (body length constant).........	0.647 ± 0.0012	0.402 ± 0.0016

When the general factors for size are eliminated by stabilizing ('26*d*) for body weight in the case of the systemic weight relations, and for body length in the case of the systemic length relations, by the method for partial correlation, the values in Table II. are obtained.

It is evident that in both sexes the systemic correlation in weight is greater than the systemic correlation in length. That is to say the reduction from the zero order value is greater in the length than in the weight relations. This signifies that in the growing animal systemic association in length is more dependent on the general size factors for length, than is the systemic association for weight dependent on the general size factors for weight. This is consistent with the fact that the growth capacity of the bones in weight differs more in type and degree from the growth capacity of the body in weight, than does the growth capacity of the bones in length differ from that of the body in length. It falls in line with the interpretation that growth of the bones in length is less systemically characteristic than growth in weight; and that growth in bone length is more closely allied to body growth in length, than bone growth in weight is allied to body growth in weight.

The table also shows that systemic association in both weight and length is greater in the male than in the female. This indicates that in the growing animal systemic association is more dependent on general size factors in the female than in the male. The relation is consistent with the fact shown on the chart that the degree of difference between the growth capacity of both bones in weight and length, and that of the body, is greater in the male than in the female.

SUMMARY AND CONCLUSIONS.

An evaluation of systemic and sex factors as determinants of the type and rate of growth of the humerus and femur of the albino rat has been made.

The results of the analysis indicate that the influence of the systemic factors is largely expressed as differences in degree, while that of the sex factors is exhibited as differences in the course as well as in the rate of growth.

From the point of view of differential development, systemic correlation is closer than sex correlation, both in rate and direction of change, particularly during the period of active differentiation (23 to 65 days of age). From this it is concluded that the sex factors of influence, which apparently are gonadal in immediate origin, are superimposed upon the systemic determinants and direct the sex-specific course of development.

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BIOLOGICAL BULLETIN

THE PRECIPITIN REACTION IN THE STUDY OF ANIMAL RELATIONSHIPS.

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GENERAL HISTORICAL INTRODUCTION.

The precipitin reaction was discovered by Rudolph Kraus in 1897. Having injected a goat with sterile cholera culture filtrates, he found that the blood serum of the goat had come to possess the property of causing a precipitate to appear when it was mixed with perfectly clear solutions of cholera culture filtrates. Normal goat serum, of course, caused no such precipitate. Furthermore, the serum of the goat injected with cholera filtrates (cholera antiserum) caused no precipitate when mixed with bacterial filtrates other than cholera. Similar results were obtained when typhoid and plague culture filtrates were used for inoculation. In these cases typhoid antiserum caused a precipitate only in typhoid filtrates and plague antiserum only in plague filtrates. The reaction was therefore specific in that a precipitate occurred only when an antiserum was mixed with a filtrate of the particular kind of bacteria used for immunization. The substances developed in the blood serum of the immunized animal were called "precipitins" and from them the reaction got its name. The materials which were injected into animals for the purpose of inducing precipitin formation were called "precipitinogens."

The precipitin reaction became of general biological significance two years later when Bordet (1899) and Tchistovitch (1899) independently found that proteins other than bacterial could act as precipitinogens, *i.e.*, could induce the formation of precipitins when injected into animals. Bordet injected fowl blood into rabbits and rabbit blood into fowls and obtained specific antisera in either case. Tchistovitch injected eel serum and horse serum into rabbits and obtained likewise antisera which gave precipitins when mixed with the particular serum used for injection. From these and many similar results obtained by other workers it was learned that in general any foreign protein was capable of stimulating precipitin formation when injected into animals.

Now such a specific reaction might be applied to the solution of many biological and medical problems. It might be used to identify unknown bacteria, to diagnose an obscure disease, to detect the adulteration of meats, or to identify unknown blood stains in medico-legal cases. Indeed, the precipitin reaction has been used for all these purposes and for others as well. The amount of work done in the solution of these problems is considerable, and the literature dealing with them is extensive. It is desired here, however, to mention only the more important contributions to problems primarily zoölogical.

To Uhlenhuth belongs the greater part of the credit for having worked out a dependable method for the identification of unknown blood stains in criminal cases. But in these early studies, Uhlenhuth (1901) records results of importance to the zoölogist. It may be recalled that the precipitin reaction, as first known, was entirely specific. Uhlenhuth noted, however, that the reaction was not always strictly specific but that sometimes an antiserum against one kind of protein would react not only with this same protein (homologous reaction) but would also react, though to a less degree, with other closely related proteins as well (heterologous reaction). The precipitin reaction was thus proven to be not strictly specific though in fact it remained quantitatively so inasmuch as the homologous reaction was always stronger than any heterologous reaction with the same antiserum. Uhlenhuth then followed up this discovery with studies on the reactions of various egg albumins and pointed out the value of studies of this kind on related proteins.

By far the most important studies on related proteins have been made by Nuttall (1904). In his book "Blood Immunity and Blood Relationship," Nuttall published the results of 16,000 tests made with thirty different antisera on 900 species of animal blood. Besides these qualitative tests there were included also 500 quantitative tests on various bloods. As a result of these tests Nuttall was able to state that the degree of reaction between an antiserum and various proteins was in proportion to the degree of relationship of these proteins to each other. Here, then, was a new method of measuring the "blood relationships" of animals, and the results obtained by Nuttall confirmed in general the existing classification of these animals based chiefly on comparative anatomy and embryology.

Since the outstanding works of Uhlenhuth and of Nuttall there have been many other contributions to the study of animal relationships based on precipitin tests. Hektoen, though interested primarily in the medico-legal aspects of the precipitin reaction, has published numerous results which have a direct bearing on problems of specificity and animal relationships. Following up the discovery of Uhlenhuth that the lens proteins of the Vertebrate eye are remarkably similar throughout, Hektoen (1922) has made many tests confirming Uhlenhuth's statements. Furthermore, Hektoen (Hektoen and Schulhof, 1924) has found that there are two antigenically distinct proteins in the crystalline lens and that apparently the lenses of all the chief groups of Vertebrates have these two elements in common. Hektoen (Hektoen and Welker, 1924) has also found that the blood serum of animals can be divided into several specific protein fractions. He has shown (Hektoen and Manly, 1923) that the semen of animals gives specific and group reactions, and that the same is true of their hemoglobins (Hektoen and Schulhof, 1923). Throughout his work he has repeatedly succeeded in obtaining specific precipitin reactions. The milk of mammals has been shown to give much the same results in the study of relationships as the blood serum.

In all this work there has been a fair degree of uniformity in results obtained. But there have been many other studies of more uncertain value. Dervieux (1921) in working with an anti-human semen serum claimed to have found an individual specificness to the reaction. Geyer (1913) states that he could distinguish between male and female Lepidopteran gonads by means of this test. Many other attempts have been made to show an organ specificity for the precipitin reaction but so far all this work has failed of confirmation, except in the case of the lens of the Vertebrate eye.

As a matter of fact even the results of Uhlenhuth, Nuttall, and Hektoen have not gone unchallenged. Strube (1902), Kister and Wolff (1902), and many others claimed that the precipitin reaction was not always specific even in the quantitative sense. And in more recent years similar claims have again been made. Thus Friedberger and Collier (1919) state that an antiserum against one kind of protein may react with equal or even greater intensity with non-related proteins. A year later Friedberger and Jarre (1920) reported upon "aspecific antisera" and claimed that many of the antisera which they produced reacted without regard to the relationship of the proteins used. Reeser (1919) independently in Holland claims the same thing and is confirmed by Yu (1923) and again by Friedberger and Meissner (1923) in Germany.

Thus the matter stands to-day, the value of the precipitin reaction in the study of animal relationships being by no means completely established. For if the precipitin reaction does not take place according to the relationship of the proteins tested, as Friedberger claims, its results will always be of uncertain value in such studies.

One more problem that has been attacked with the aid of immune reactions including the precipitin test should be mentioned. This is in the field of experimental evolution and the work referred to is that of Guyer and Smith (1920). By injecting antisera against rabbit lens into pregnant rabbits these investigators have obtained hereditary eye defects which appeared in many succeeding generations. Furthermore, just recently Guyer (1925) has been able to demonstrate that by needling the lens of rabbits lens defects may be caused in their young. The significance of these discoveries in the solution of problems of experimental evolution may be almost unlimited.

The precipitin reaction has then contributed something to the solution of at least two fundamental zoölogical problems, viz., animal relationships and evolution. It would seem perhaps worth while to make a careful study of the reaction in the hope of clearing away discordant results and of properly evaluating the reaction as a research tool. And inasmuch as precipitins are little known to zoölogists in general it has seemed advisable to explain the reaction more fully than would otherwise be necessary.

PURPOSE OF THIS INVESTIGATION.

The purposes of this investigation are (1) to study the accuracy and the dependability of the precipitin reaction and factors which may increase these qualities, (2) to apply an improved technique of the reaction with a known per cent. of error to the study of animal relationships.

TECHNIQUE.

Historical Review.

As first performed by Kraus in 1897 the precipitin reaction consisted in the mixing of a sterile antiserum (formed by injecting goats with bacterial culture filtrates) with clear sterile bacterial filtrates in small glass tubes which were then placed in the incubator at 37° C. After twenty-four hours the tubes were examined and those which were markedly cloudy and showed flocculent precipitates were recorded as positive. The "titer" or strength of any antiserum was measured by putting into a series of similar tubes containing increasing dilutions of the antigen (protein to be tested) in normal salt solution, a definite amount of antiserum, then mixing, incubating, and recording after a certain definite time the highest dilution in which a precipitate occurred. As controls, tubes containing normal blood serum and antigen dilution, and tubes containing antiserum plus salt solution alone, were used.

This technique was used by many workers in the titration of a great variety of antisera and antigens. It soon came to be noticed, however, that the end point of this "flocculation" test was exceedingly hard to determine, for a cloudiness might occur in the tubes without a settled precipitate and therefore the cloudiness of the experimental tubes graded off imperceptibly into the cloudiness of the controls. The probability of error in such determinations must have been very high and no doubt some of the discordant results obtained by early investigators were due to this very fact.

Great steps in advance came with the works of Ascoli (1902) and Fornet and Muller (1908, 1910) in the development of the "ring test" and with Nuttall's quantitative measurements of the amount of precipitate formed (1902). Ascoli suggested that the antiserum be carefully layered under the antigen dilution somewhat as the Heller's nitric acid test is performed. Then in positive reactions a definite white stratum of precipitate forms at the zone of contact between antiserum and antigen, the so-called "ring," which is quite obvious and gives the reaction a sharp end point. Fornet and Muller further studied this technique and recommended this ring test strongly because of its greater definiteness. Today the best workers employ this test and indeed there is no longer any excuse for the continued use of the older flocculation test.

Nuttall published methods for making a quantitative flocculation test in 1902. His method consisted in the volumetric measurement of the amount of precipitate formed as a result of mixing a definite amount of an antiserum with a definite amount of a certain antigen dilution. Repeated tests on homologous bloods gave an error of about ten per cent. but sometimes when testing an antiserum with a heterologous blood the error reached fifty per cent. This error seems due partly to the small amounts of precipitate measured and partly to variations in the closeness with which the particles of the precipitate became packed.

The effects of temperature and of salt concentration on the titer of antisera have also been studied. Though there were some discordant results the influence of temperature was found to be well marked, the rate of precipitation increasing with temperature up to about 40° C. With regard to the effect of salt concentration there is less agreement. In general, however, increasing salt concentration decreases the amount of precipitate. Normal physiological salt solution (.85 to .9 per cent.) is generally used today. Hektoen (1918) recommends a salt solution of double the strength of normal saline for use with chicken antisera. This he states increases the specificity of the antiserum.

Recently the effect of variations in the H-ion concentration of the salt solution on the reaction has been studied. Using the flocculation test Mason (1922) found no significant effect within a range of P_H 4.5 to 9.5. However the flocculation test is not sensitive enough to show a slight effect had there been one. Evans (1922) and Mason and Sanford (1924) find that the H-ion does have an effect on various serological reactions and recommend buffered physiologic salt solutions for use in serological work.

With regard to the mode of injection of animals it has generally been found that intravenous injections give higher titers than any others. Such injections are given in increasing doses as four, six, eight cubic centimeters of serum on successive days or at two to five day intervals. The titer reaches its climax about the tenth day after the last injection. Rabbits and chickens have been found to be the most practical precipitin producers.

Material and Methods Used in this Investigation.

Rabbits and chickens have been used to produce the antisera. They have been injected with sterile blood sera of healthy sheep, goat, pig, horse and dog. The injections were usually intravenous though many were intraperitoneal. They were given for the most part at three day intervals the dosage being 4 cc., 6 cc., and 8 cc. of the particular blood serum used as antigen. Ten days after the third injection the rabbits were bled from the median ear artery and the fowls from the wing vein, a five cc. sample being withdrawn for a preliminary titration. In many cases the antisera so obtained possessed a titer of 6,400 or over. That is, a definite ring appeared in a dilution of one part of antigen in 6,400 parts of salt solution, at 1 hour, but did not appear in any greater dilutions. In other cases one or two more injections were given, the first one being 2–4 cc. and given intraperitoneally, the second 5–10 cc. and given intravenously. No symptoms of anaphylaxis have ever been noted. Usually the titer of these reinjected animals had now increased to 6,400 or over. All such titers were thought to be satisfactory and the animals possessing them were bled completely as soon as possible after the preliminary titration. Both rabbits and fowls were starved for eighteen hours preceding the final bleeding in order that the blood be as clear as possible.

The rabbits were bled from the dorsal aorta. The animals were first anaesthetized with ether and then a median ventral incision was made. The viscera were moved to one side and the dorsal aorta carefully cleaned of fat and connective tissue for a short distance. The aorta was then clamped off centrally and distally and a sterile canula inserted through a small incision and securely ligatured. The central clamp was then removed and the blood allowed to flow through sterile rubber tubing into centrifuge tubes. In this manner 75–100 cc. of blood were usually obtained.

Fowls were bled completely after decapitation, the amount of blood varying from 35–125 cc. depending upon the size of the individual.

The blood was allowed to stand for two or more hours in the ice-box after which time the clot was broken up and the blood centrifugalized. The antiserum was then poured off and stored in 5 cc. sterile, rubber-stoppered vaccine vials which were then kept in the ice-box. In many cases the antisera were filtered through small Berkfeld filters before bottling to insure sterility.

The blood sera used as antigens were obtained from healthy animals. The beef and pig bloods were obtained from the Mayer Packing Plant, the horse blood from Mr. Fred Rieder, and the sheep and goat bloods from animals kept by the Department of Zoölogy. Besides these, other bloods were used for titration only. The latter included human, rat, guinea pig, rabbit, fish (carp) and fowl. The sera were obtained after clotting and centrifugalizing and were usually filtered through Berkfeld filters before storing in 5 cc. ampules in the ice-box.

From these blood sera standard solutions were made. These standards were usually 2 or 4 per cent. dilutions of a given serum in normal saline (sometimes 10 per cent.). The standards were made in 100 cc. volumetric flasks and filtered through a Berkfeld filter and then stored in the usual manner in 5 cc. vials. The concentration of protein in each standard was determined by making modified Kjeldahls on 5 or 10 cc. samples according to the method of Folin and Wright (1919). Total nitrogen was determined on one sample and non-protein nitrogen on another, the protein in the latter being removed by precipitation by sodium tungstate (10 per cent.; 1 cc.) and sulphuric acid (2/3 N; 1 cc.). The protein nitrogen was then obtained by difference and the protein calculated (protein N x 6.25) as grams per 100 cc. serum. The titrations recorded hereinafter have all been made with these standard antigen solutions of known protein concentration.

The ring test has been employed throughout, the observations being made at room temperature. As suggested by Evans (1922) buffered salt solutions have been made according to Sorenson's tables given in Clark (1922). These buffered solutions were made to have a P_H of 7 determined colorimetrically except in certain cases where the P_H has been specially noted. The successive increasing dilutions have been made according to the usual Stern-Korte method. Thus a series of small clean glass tubes is set up in a rack and to each tube is added $\frac{1}{2}$ cc. of buffered salt solution with a sterile 10 cc. pipette. To the first tube of the series is added $\frac{1}{2}$ cc. of a standard antigen dilution using a 1 cc. pipette. The fluids are mixed by sucking up into this pipette several times. From this mixture (now $\frac{1}{2}$ as concentrated as the standard solution) $\frac{1}{2}$ cc. is transferred to the next tube, etc. Thus each tube in the series contains a concentration of protein in buffered salt solution (.85 per cent. NaCl for rabbit sera and usually 2.25 per cent. for chicken sera) one half as great as the preceding tube. The antisera used were generally of a strength of about 1 : 10,000 which requires eight tubes beginning with a 2 per cent. standard solution. Now to each of these dilutions beginning at the highest is added .1 cc. of the antiserum to be titrated the pipette being placed in the bottom of the tube and the antiserum carefully layered under the antigen dilution. As a control .1 cc. of the same antiserum is layered under $\frac{1}{2}$ cc. of buffered salt solution. The tubes are then allowed to stand at room temperature and read at 20, 40, and 60 minutes by light from an electric bulb transmitted through a narrow horizontal slit behind the tubes.

When a suitable antiserum has finally been obtained preliminary titrations are first made with the homologous antigen and then with various heterologous antigens in the manner just described. But in all cases in which sufficient antiserum was available these preliminary titrations were followed by one or more double titrations made in the following manner.

One series of tubes was set up with dilutions starting from a 2 per cent. standard solution. A second series was then set up starting with a dilution of 1 : 150 of this same antigen. This was made by mixing $\frac{1}{2}$ cc. of 2 per cent. solution (1 : 50) with 1 cc. of salt solution. By using this double series of tubes the difference between the concentration of one tube and its nearest successor was reduced to 50 per cent. instead of 100 per cent. See Fig. I.

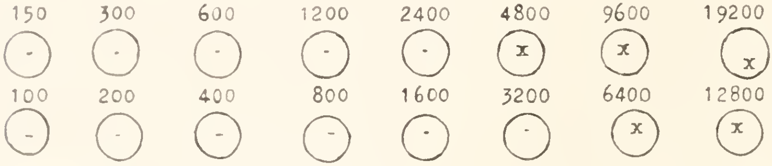

FIGURE 1. A diagram to illustrate the double series of dilutions employed in titration.

Now if the preliminary titration had given a titer of 6,400, in the final titration only the tubes from 4,800 to 19,200 (marked with an x in Fig. 1) would be given antiserum. The reading in the final titration falls generally within one tube of the preliminary titration. By using this double titration a more accurate value of the titer is obtained without the need of using excessive amounts of antiserum. The tubes containing antiserum were then put in order of increasing dilution in a rack and read as described.

EXPERIMENTAL RESULTS.

RESULTS ON TECHNIQUE.

1. *The Range of Error in Reading the Ring Test.*

Experimental results of any kind whatever have significance only insofar as it is definitely known that they pass beyond the range of experimental error. The first step in the application of the precipitin reaction to biological problems should then consist in the establishment of the range of error in reading the reaction for each investigator.

Rabbit P41 was injected with goat serum. The antiserum so obtained was repeatedly titrated against standard goat antigens at a P_H of 7. A double series of dilutions was made so that the succeeding tubes in the series had concentrations of antigen 50 per cent. less than their predecessors. The results of twelve such titrations are shown in Table I.

TABLE I.

RANGE OF ERROR IN READING THE RING TEST.

Antigen 2 Per Cent. Goat Serum, Antiserum P41.

Number of Test.	Order of Tubes.	4,800.	6,400.	9,600.	12,800.	Time.
1	Known	+	−	−	−	20 minutes
2	“	+	−	−	−	20 “
3	“	+	+	−	−	20 “
4	“	+	+	−	−	20 “
5	“	+	+	−	−	20 “
6	“	+	+	−	−	20 “
1	“	+	+	−	−	40 “
2	“	+	+	−	−	40 “
3	“	+	+	+	−	40 “
4	“	+	+	+	−	40 “
5	“	+	+	+	−	40 “
6	“	+	+	+	+	40 “
1	“	+	+	+	−	60 “
2	“	+	+	+	+	60 “
3	“	+	+	+	+	60 “
4	“	+	+	+	+	60 “
5	“	+	+	+	+	60 “
6	“	+	+	+	+	60 “
1	Unknown	+	−	−	−	20 “
2	“	+	−	−	−	20 “
3	“	+	+	−	−	20 “
4	“	+	+	−	−	20 “
5	“	+	+	−	−	20 “
6	“	+	+	−	−	20 “
1	“	+	+	−	−	40 “
2	“	+	+	−	−	40 “
3	“	+	+	+	−	40 “
4	“	+	+	+	−	40 “
5	“	+	+	+	−	40 “
6	“	+	+	+	−	40 “
1	“	+	+	+	−	60 “
2	“	+	+	+	−	60 “
3	“	+	+	+	−	60 “
4	“	+	+	+	−	60 “
5	“	+	+	+	+	60 “
6	“	+	+	+	+	60 “

Table showing 12 titrations of the same antiserum and antigen using a double series of tubes and dilutions.

The results shown in Table I. are (1) that readings of the ring test generally corresponded at a given period, (2) that the titer increased with time up to an hour, (3) that the greatest deviation in the reading was plus or minus one tube or 50 per cent. in terms of titer, (4) that when the order of the tubes was that of increasing dilutions and known to the reader a higher reading was usually obtained than when the order was irregular and unknown. This latter result is natural for if the tubes are arranged in order of increasing dilutions of antigen, the rings occur strongly in the lower dilutions and gradually less strongly in the higher dilutions and the first tube to be apparently negative often on close observation will be seen to possess a faint but definite ring. This might easily be overlooked if the order of the tubes is unknown. For this reason the order of the tubes has been known in all titrations on relationships.

Now a possible error of plus or minus 50 per cent. in reading the ring test (which increases to 100 per cent. when a single series of dilutions is used) doubtless seems very high but one must remember that this is a biological test and that in spite of this error proteins may be identified in dilutions far beyond the range of any chemical method known at present. By "possible error" is meant simply that inasmuch as the dilutions tested were each 50 per cent. less than the preceding dilution, the maximum error, should the end point actually fall somewhere between two successive dilutions, would be 50 per cent. It is to be remembered that this possible error is inherent only in the serological titration not in the preparation of the antigen solution (error less than 4 per cent. in nitrogen determinations). The probable error would not be more than half as great as this possible error. It is felt that the use of a double series of dilutions is justified on account of the more accurate reading thus obtained. A triple or quadruple series of dilutions might be used with an even greater accuracy but the labor involved in their use is so great as to preclude their practice.

2. *The Sensitivity of the Ring Test.*

Fifteen antisera have been carefully titrated with standard antigens. Their homologous titers at one hour were distributed as follows:

Titer or Dilution	4,800	9,600	12,800	19,200	25,600	76,800	102,400	204,800
No. of Antisera.......	1	1	3	3	4	1	1	1

The concentration of antigen in these dilutions being known, it is possible to calculate the amount of protein present in the last tube in which a positive reaction occurred. The antiserum with a titer of 4,800 positively identified the presence of .0088 mgms. of goat serum. The most powerful antiserum produced identified sheep serum when less than .0002 mgms. of protein was present. The other antisera fell in between these limits. Needless to say even the weakest antiserum employed was far more sensitive to a specific protein than any chemical method known. Much of the value of the precipitin reaction in research is based on this great sensitivity.

TABLE II.

THE EFFECT OF ANTIGEN CONCENTRATION ON TITER.

Time.	Antigen.	3,200.	4,800.	6,400.	9,600.	12,800.
20 minutes...	1%	+	–	–	–	–
20 " ...	1%	+	–	–	–	–
20 " ...	1%	+	–	–	–	–
20 " ...	1%	+	–	–	–	–
20 " ...	2%	+	+	–	–	–
20 " ...	2%	+	+	–	–	–
20 " ...	2%	+	+	+	–	–
20 " ...	2%	+	+	+	–	–
20 " ...	2%	+	+	+	–	–
20 " ...	2%	+	+	+	–	–
40 " ...	1%	+	–	–	–	–
40 " ...	1%	+	+	–	–	–
40 " ...	1%	+	+	–	–	–
40 " ...	1%	+	+	–	–	–
40 " ...	2%	+	+	+	–	–
40 " ...	2%	+	+	+	–	–
40 " ...	2%	+	+	+	+	–
40 " ...	2%	+	+	+	+	–
40 " ...	2%	+	+	+	+	–
40 " ...	2%	+	+	+	+	–
60 " ...	1%	+	+	–	–	–
60 " ...	1%	+	+	–	–	–
60 " ...	1%	+	+	–	–	–
60 " ...	1%	+	+	+	–	–
60 " ...	2%	+	+	+	+	–
60 " ...	2%	+	+	+	+	–
60 " ...	2%	+	+	+	+	–
60 " ...	2%	+	+	+	+	–
60 " ...	2%	+	+	+	+	+
60 " ...	2%	+	+	+	+	+

3. *The Effect of Antigen Concentration on the Titer of Antisera.*

Table II. shows the result of titrating the same antiserum (P41) with two homologous standard antigens of different strengths. All tubes were unknown in this experiment.

The results given in Table II. show that the titer of an antiserum varies directly with the concentration of the antigen used. Indeed it has long been known in a general way that such was the case. It has also been known that various antisera differ in titer when tested with the same homologous antigen. There are therefore two chief factors in this reaction, (1) the concentration of antigen, (2) the concentration of antibody in the antiserum. Now of these two the latter is but partly controllable. For we may inject various amounts of protein into animals but we must take whatever titer of antibody they yield. On the other hand the concentration of antigen can be definitely controlled and this should be made the standard of comparison for all workers in this field. For this reason only standard antigen solutions have been used in this investigation.

The results of the nitrogen determinations on the standards used are given in Table III.

From the data given in Table III. it may be seen: (1) that the blood sera used in general possessed about the same protein concentration, but that the dog serum was enough lower than the average to affect definitely the titer of its reactions, (2) that the amount of non-protein nitrogen in the fresh blood sera used was generally so small as to be well within the range of error of the titration, (3) that the amount of non-protein nitrogen generally increased in the standard solutions which were sometimes kept for several months. The values for non-protein nitrogen in these older standards sometimes approached a level high enough to become of consequence in the comparative titration of heterologous antigens.

Probably in fresh sera the error due to calculation of protein from total nitrogen would seldom exceed 10 per cent. Total nitrogen determinations alone would then give figures sufficiently accurate for comparative studies. It is hoped that the users of the precipitin reaction in the study of related proteins or in other quantitive studies will feel the necessity of getting comparable results and will come to make total nitrogen determinations on all antigens used in titrations. Perhaps it would be well to speak of the latter as secondary antigens to distinguish them from proteins injected into animals for the purpose of inducing antibody formation which would then be called primary antigens.

Table III.

Nitrogen Determinations on Standard Solutions.

Standard.	Date of Preparation.	Total N Grams per 100 cc. Serum.	Date of Determination.	Non-protein N in mgms. per 100 cc. Serum.	Date of Determination.	Protein in gms. per 100 cc. Serum.	% of Non-protein N in Total Nitrogen.
Beef serum							
No. 1–2 %	1–6–25	1.295	1–7–25	51	1–13–25	7.736	4.4
" 2–2 %	1–18–25	1.365	1–29–25	10	1–29–25	8.468	.74
" 3–2 %	4–20–25	1.176	4–28–25	trace	4–28–25	7.350	
Dog serum							
4 %	2–23–25	.805	5–1–25	119	5–1–25	4.290	14.8
Goat serum							
No. 1–2 %	1–11–25	1.2859	1–13–25	87.5	1–13–25	7.487	7.4
" 2–2 %	3–24–25	1.120	4–28–25	140	4–28–25	7.925	12.5
Horse serum							
4 %	2–23–25	1.085	5–1–25	61.25	5–1–25	6.394	5.6
Human							
serum 2 %	1–19–25	1.260	1–19–25	32.9	1–19–25	7.668	2.6
Pig serum							
No. 1–2 %	1–6–25	1.309	1–7–25	145.25	1–14–25	7.275	11.0
" 2–4 %	2–27–25	1.185	5–1–25	94.5	5–1–25	6.812	8.0
Sheep serum							
No. 1–2 %	1–6–25	1.232	1–7–25	96.25	1–13–25	7.100	7.7
" 2–2 %	3–8–25	1.330	5–1–25	140.0	5–1–25	7.438	10.9
Tame rat							
2 %	1–4–25	1.575	5–1–25	49.7	5–2–25	9.097	7.7

Now when proteins other than fresh sera are used as secondary antigens, the necessity of determining their concentration is very much greater. For the concentration of protein in the sera of animals which have died of various diseases (so often used by Nuttall), or in extracts of filter paper soaked in blood and then dried, or in extracts of blood stains from various objects, or in extracts of tissues, varies exceedingly. This it seems was the chief source of error in Nuttall's work as it has also been in the work of many others. For heretofore nothing but a foam test, or a crude nitric acid boiling test have been used in the determination of antigen concentration. The precipitin reaction seems worthy of better technique than this.

4. *The Effect of the Time of the Reading on Specificity.*

Since the titers (both homologous and heterologous) of fifteen antisera have been recorded at twenty, forty, and sixty minute intervals, the effect of time on the specificity of the reactions can be shown. The results are given in Table IV.

TABLE IV.

RELATIVE SPECIFICITY OF READINGS AT 20, 40, AND 60 MINUTES.

Anti-serum.	Antigen.	Heterologous Reactions in % of Homologous Reaction.		
		20 min.	40 min.	60 min.
A21 anti-pig	Pig	100 %	100 %	100 %
	Beef	12.5	8.3	12.5
	Horse	6.6	8.3	9.4
	Goat	6.6	6.6	6.25
	Sheep	4.7	6.6	6.25
	Dog	4.7	6.6	4.7
	Human	2.4	2.1	3.13
A24 anti-beef	Beef	100	100	100
	Sheep	37.5	50.0	50.0
	Goat	37.5	50	50
	Pig	9.4	25	16.6
	Horse	12.5	25	25
	Dog	9.4	18.8	16.6
	Human	12.5	18.8	12.5
P10 anti-sheep	Sheep	100	100	100
	Goat	66	100	75
	Beef	33.3	50	50
	Pig	1.04	2.08	3.1
	Horse	0.52	2.08	3.1
	Dog	1.04	2.08	3.1
	Human	0.52	0.52	0.58

The three antisera whose reactions are given in Table IV. have been taken at random from the entire number and are believed to be typical of them all. Of the eighteen heterologous reactions recorded fourteen showed a greater specificity at twenty minutes than at forty minutes or one hour. That is, the heterologous reactions at twenty minutes were proportionally less compared with the homologous reactions at the same time than at any later time. Those facts are of significance, for in medico-legal work where the greatest possible specificity is desired the earlier reading is better, whereas in the study of relationships a later reading is preferable. Experience has shown that one hour is the latest time at which the ring test may be read satisfactorily at room temperature. After that time the rings become diffuse and tend to disappear.

5. *The Effect of P_H on the Titer of an Antiserum.*

An antibeef serum (A8) was titrated at several different P_H values as shown in Table V. The buffered solutions were made according to Sorenson's tables as recommended by Evans (1922) and were tested colorimetrically after sterilization in the autoclave. The homologous readings at one hour are given in the table.

TABLE V.

THE EFFECT OF P_H ON TITER.

P_H.	3,200.	4,800.	6,400.	9,600.	Control.
5.5	+	±	±	±	±
5.5	+	±	±	±	±
6.0	+	−	−	−	−
6.0	+	+	−	−	−
6.0	+	+	+	−	+
6.0	+	+	±	±	±
7.0	+	−	−	−	−
7.0	+	−	−	−	−
7.0	+	+	−	−	−
7.0	+	+	−	−	−
8.0	+	−	−	−	−
8.0	+	−	−	−	−

From the data given in Table V. it may be seen that a spontaneous precipitate often appeared in the controls below a P_H of 7. The titer of the antiserum was not affected in any marked way except through the effect on the control. Such a spontaneous precipitate in the control must be prevented and this alone justifies the use of buffered solutions in the precipitin test. Other workers claim even greater effects of the P_H on serological reactions.

6. *The Effect of Sodium Chloride Content on Titer.*

The titrations of rabbit antisera have all been made with .85 per cent. buffered saline with a P_H of 7 except in special experiments. It was noted, however, that certain chicken antisera gave spontaneous rings in such salt solutions. It became necessary then to inhibit this reaction in order to record ring tests due to the presence of antigen in the salt solution. Following a suggestion of Hektoen's (1918) a more concentrated salt solution was used. It was found that in some cases a 2.25 per cent. salt solution was necessary to inhibit completely this spontaneous ring. The question then arose as to whether such a heavier salt concentration would alter the group reactions of a given antiserum. To settle this matter a chicken antiserum which did not require a 2.25 per cent. saline was nevertheless titrated with the same series of antigens in both .85 per cent. and 2.25 per cent. saline. The results are shown in Table VI.

TABLE VI.

THE EFFECT OF NACL CONTENT ON TITER.

Antiserum.	Salt Concentration.			
	.85% Titer.	PH 7 Per Cent.	2.25% Titer.	PH 7 Per Cent.
C 8 (antibeef) Beef	102,400	100 %	25,600	100 %
Sheep	102,400	100	12,800	50
Goat	102,400	100	12,800	50
Pig	12,800	12.5	800	3.1
Horse	6,400	6.25	800	3.1
Dog	3,200	3.1	800	3.1
Human	3,200	3.1	400	1.6

Table VI. shows the distinct inhibition due to the heavier salt solution. Furthermore the specificity of the antiserum has been increased. As a matter of fact the range of reactions in chicken antisera is more closely parallel with that of rabbit antisera when this heavier salt solution is used (see Figs. 3 and 5).

7. *Effect of Filtration on Titer.*

When an antiserum becomes contaminated it must be filtered at once for the titer rapidly decreases during putrefaction. The possible effect of such filtration on the specificity of reaction of antisera must then be known. For this purpose parallel titrations have been made on both rabbit and chicken antisera before and after filtration. The results are shown in Table VII.

The results given in Table VII. are typical of them all with the exception of a single chicken antiserum which apparently lost some of its antibodies on filtration. Small Mandler filters have been used for this filtration. In general there has been no loss of titer. In fact the titer usually increases after filtration due to the increased clarity of the antiserum and the consequent greater ease of detecting a fine ring in the last tube. Insofar as the effect on specificity has passed beyond the range of error it has been in the direction of a lesser specificity and would thus tend to neutralize the effect of using 2.25 per cent. salt solutions in the chicken antisera.

TABLE VII.

THE EFFECT OF FILTRATION ON TITER.

Antiserum.	Antigen.	Unfiltered.		Filtered.	
		Titer.	Per Cent.	Titer.	Per Cent.
P123 (antidog)	Dog	19,200	100 %	25,600	100 %
	Beef	800	4.7	1,200	4.7
	Horse	400	2.4	600	2.4
	Sheep	200	1.2	600	2.4
	Goat	200	1.2	400	1.6
	Pig	100	.6	200	.8
	Human	100	.6	400	1.6
C17 (antisheep)	Sheep	51,200	100	76,800	100
	Beef	19,200	37.5	25,600	33.3
	Horse	800	1.56	800	1.04
	Pig	000	0.00	200	.26
	Dog	100	.2	100	.13
C11 (antisheep)	Sheep	19,200	100	25,600	100
	Goat	12,800	66.66	25,600	100
	Beef	6,400	33.33	19,200	75
	Dog	1,600	8.33	3,200	12.5

RESULTS ON RELATIONSHIPS.

The experimental results bearing on matters of technique have all been secondary to the main problem of this investigation, viz., the application of the precipitin reaction to the study of of the relationships of certain animals. In the tables and figures recording the data obtained in the study of relationships the homologous reaction is given as 100 per cent. and the heterologous reactions in per cent. of the homologous titer for each antiserum and antigen. The results are given in Tables VIII. to XIII. A part of these results are given in graphical form in Figs. 2 to 8. In these figures the ordinates represent antigen dilution, or titer, given in the geometric progression of the increasing dilutions employed. The homologous reaction is given first in order from left to right along the abscissa and then follow heterologous reactions in the order of relationship to the homologous protein.

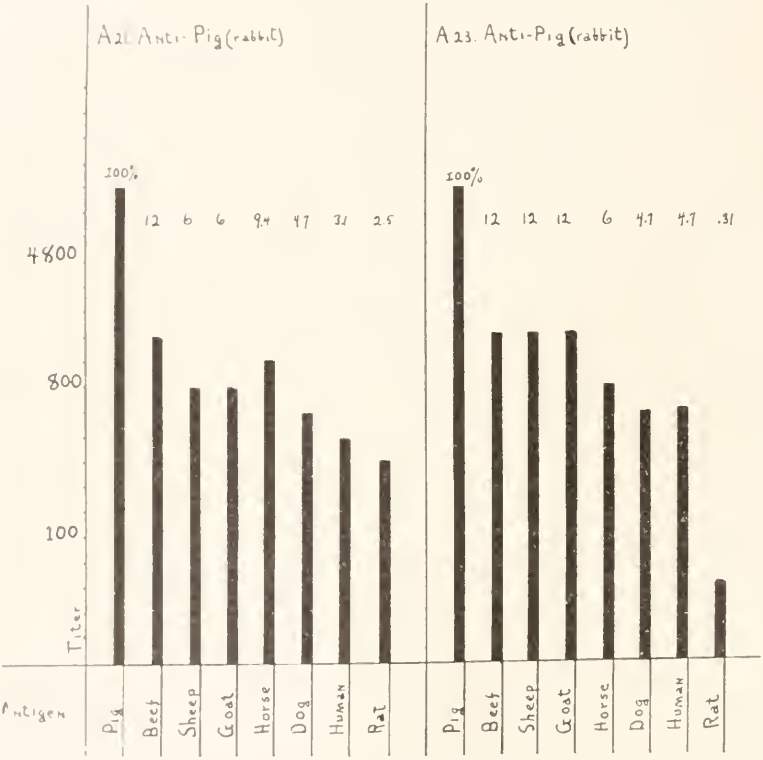

FIG. 2. Comparison of two different anti-pig sera from rabbits. These antisera were of the same homologous titer.

Table VIII. gives the reactions of three different antipig sera, two of which were obtained from rabbits and one from a fowl. A21 and C4 were injected with the blood of the same pig, A23 with the blood of another pig. These three antisera were of

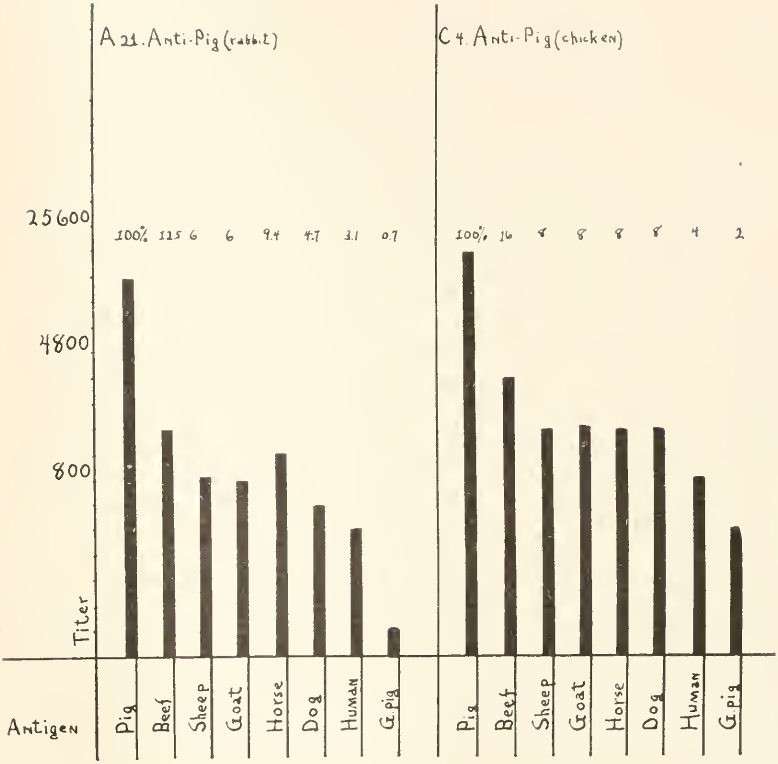

FIG. 3. Comparison of two anti-pig sera of nearly the same strength, one from a rabbit, the other from a fowl.

nearly the same homologous titer. The results are plainly shown in graphical form in Figs. 2 and 3. Considering that the range of error in reading the ring test was 50–100 per cent. there

TABLE VIII.

REACTIONS OF ANTIPIG SERA.

Antigen.	A21 Antipig Rabbit Unfiltered.	A23 Antipig Rabbit Filtered.	C4 Antipig Chicken, Unfiltered 2.25% Saline.
Pig	100 %	100 %	100 %
Beef	12.5	12.5	16.66
Sheep	6.25	12.5	8.33
Goat	6.25	12.5	8.33
Horse	9.4	6.25	8.33
Dog	4.7	4.6	8.33
Human	3.13	4.6	4.16
Rat	2.5	.31	
Guinea pig	.7		2.08
Rabbit			1.04

seems to be essential agreement in the reactions of these sera. This is true even though the antisera were produced in such different animals as rabbits and fowls.

TABLE IX.

REACTIONS OF ANTIBEEF SERA.

Antigen.	A22 Antibeef Rabbit Filtered.	A24 Antibeef Rabbit Before Filtering.	C8 Antibeef Chicken, Filtered .85% Saline.
Beef	100 %	100 %	100 %
Sheep	66	50	100
Goat	50	50	100
Pig	16.66	8.33	12.5
Horse	16.66	8.33	6.25
Dog	16.66	8.33	3.1
Human	8.33	8.33	3.1
Wild rat	.2	.1	1.6

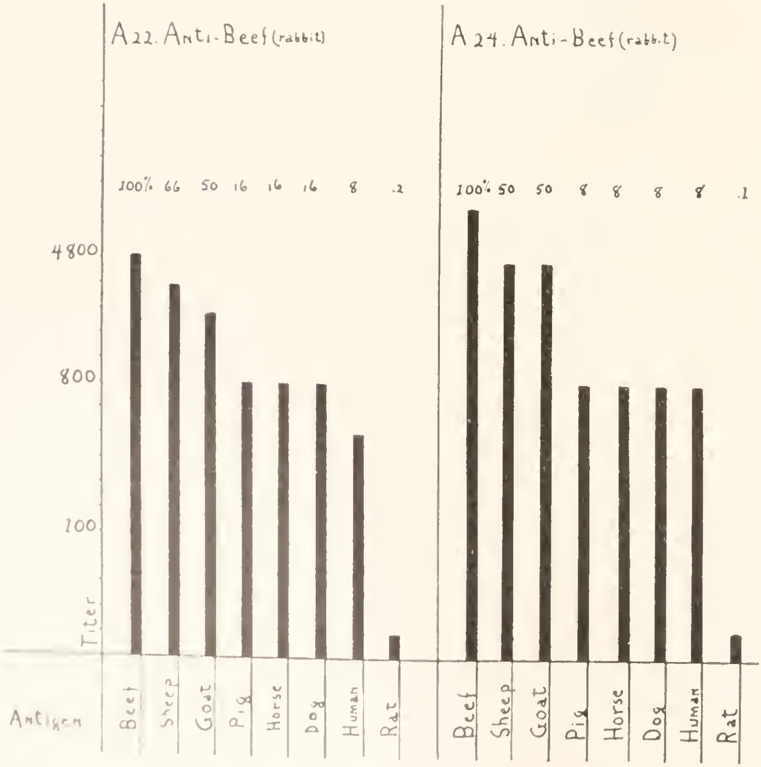

FIG. 4. Comparison of two different anti-beef sera from rabbits.

The animals whose sera are shown are usually considered to be related to each other as follows: pig, beef, sheep and goat belong to the order Artiodactyla; horse belongs to the closely related order Perissodactyla; dog, human and guinea pig belong to the distantly related orders, the Carnivora, Primates and Rodentia respectively. Except for the horse the reactions all correspond to the accepted classification of these animals.

Table IX. and Figs. 4 and 5 show the results obtained with three antibeef sera. Two of these were obtained from rabbits, the third from a fowl. A24 and C8 were injected with blood serum from the same cow. A22 was injected with serum from two different cows. The homologous titers of these antisera were not the same. In spite of this there is again a general correspondence in results obtained with one important exception. The latter is the rodent reaction. Whereas a very low value for rodent blood was given by the rabbit antisera, a very much higher value was obtained in the case of the chicken antiserum. No one has ever been able to produce a rabbit antiserum which would react against rabbit blood. There is therefore an inhibition of precipitin formation in a rabbit when injected with rabbit blood. This same inhibition would be expected to extend to animals closely related to rabbits, that is to other rodents at least. No such inhibition for rodent blood would be expected in chicken antisera and indeed as the data show a much greater reaction is then obtained. It seems therefore that chicken anti-

TABLE X.

REACTIONS OF ANTISHEEP SERA.

Antigen.	P37 Antisheep Rabbit Filtered.	P40 Antisheep Rabbit Filtered.	C11 Antisheep Chicken Filtered, 2.25% Saline.	C17 Antisheep Chicken Filtered, 2.25% Saline.
Sheep.....	100 %	100 %	100 %	100 %
Goat......	66	75	100	66.66
Beef......	50	50	75	33.33
Pig.......	8.5	3.1	0.0	.26
Horse.....	4.16	3.1	0.00	1.04
Dog......	4.16	3.1	12.5	.13
Human ...	4.16	.58	0.00	
Tame rat..			0.00	

sera would give us a truer picture of the relationships of rodents to other mammals than would rabbit antisera.

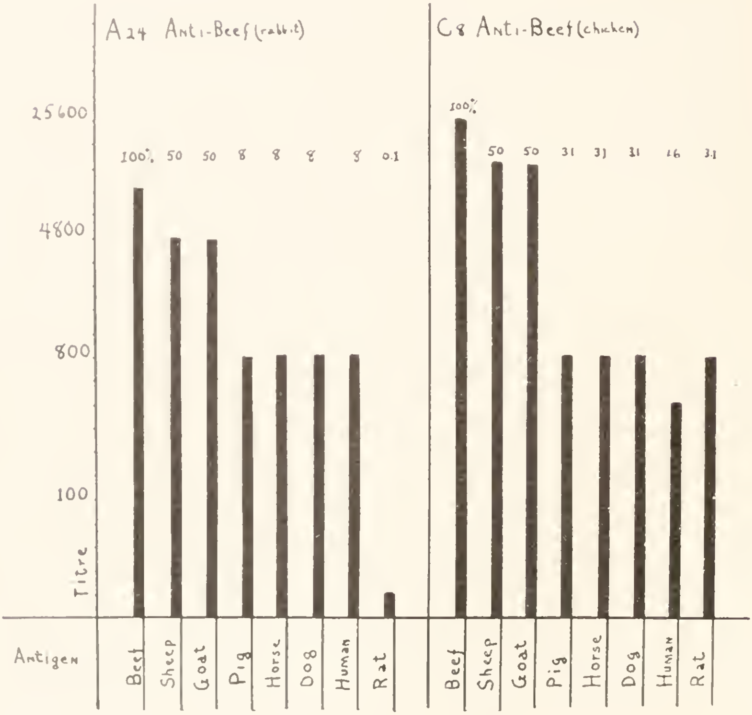

FIG. 5. Comparison of two anti-beef sera, one from a rabbit, the other from a fowl.

Table X. shows the results obtained with four antisheep sera, two obtained from rabbits and two from fowls. Fig. 6 gives a comparison of the two rabbit antisheep sera. While there is a good correspondence in the two sera obtained from rabbits, and this in spite of a great difference in their strength, there is not such a good correspondence in the chicken sera. Some of the values obtained with the chicken sera correspond closely with the others but especially in C11 no reaction for pig, horse, and human blood was obtained at all. Apparently this chicken produced no antibodies for these bloods. This seems evidence for a multiplicity of antibodies present in an antiserum produced in response to the injection of a single blood serum.

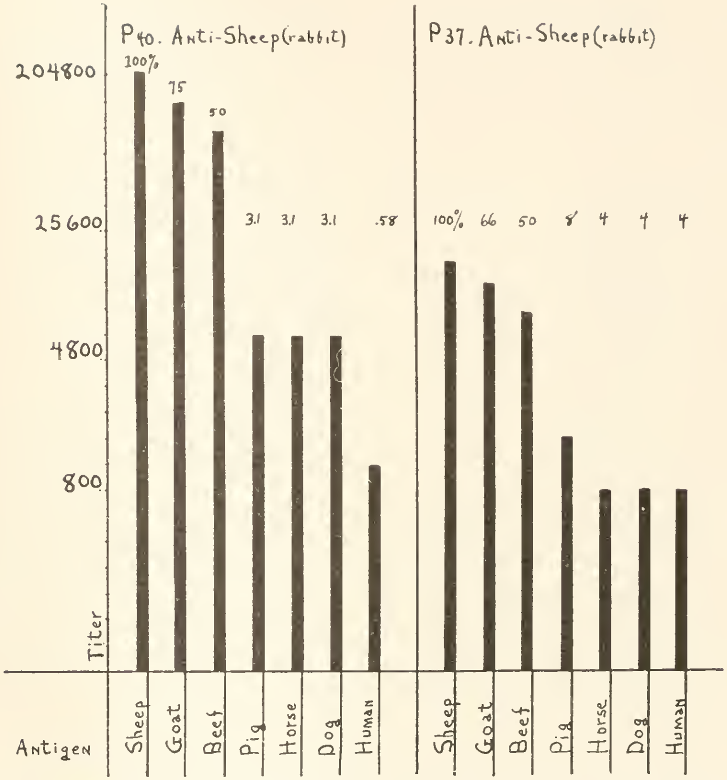

FIG. 6. Comparison of the reactions of two anti-sheep sera from rabbits, the homologous titers being quite different.

TABLE XI.

REACTIONS OF ANTIDOG SERA.

Antigen.	P123 Antidog Rabbit Filtered.	P140 Antidog Rabbit Filtered.
Dog	100 %	100 %
Beef	4.7	12.5
Horse	2.4	12.5
Sheep	2.4	12.5
Goat	1.6	9.4
Human	1.6	9.4
Pig	.8	9.4
Wild rat	0.00	6.25

Table XI. and Fig. 7 give the reactions of two antidog sera obtained from rabbits. It may be seen that the correspondence between these sera is not as close as with the rabbit sera given before. This is true in spite of the fact that both rabbits were injected with the blood from a single dog.

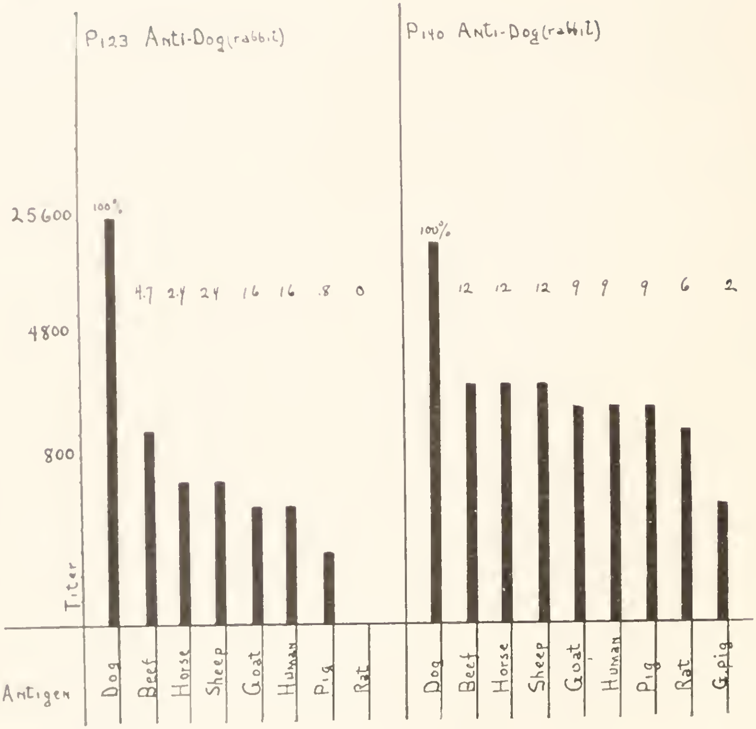

FIG. 7. Reactions of two anti-dog sera from rabbits.

Table XII. shows the reactions obtained with four different antihorse sera, two from rabbits and two from fowls. The blood for these injections was obtained from a single horse. The rabbit sera are shown in Fig. 8. As may readily be seen the poorest correspondence in the whole series of antisera was obtained here.

The data for all these reactions show that there may or may not be correspondence in the reactions of various antisera of the same kind. If this is true how may it ever become reasonably certain that any series of values represents the true relationships of the animals tested? Perhaps there are two criteria by which we may recognize the truth in these reactions. In the first place where a general correspondence occurs in the reactions of two or more antisera of the same kind, produced in similar or different animals, it would seem probable that the reactions represented some reality in the relationships of the animals tested. This would be especially true where an antiserum produced in a rabbit corresponded closely with one produced in a chicken. In the second place, the relative relationship of two different animals should be the same whichever one of these is used as primary antigen. For instance pig should bear the

TABLE XII.

REACTIONS OF ANTIHORSE SERA.

Antigen.	A25 Antihorse Rabbit Filtered.	P105 Antihorse Rabbit Filtered Twice.	C14 Antihorse Chicken Unfiltered, 2.25% Saline.	C18 Antihorse Filtered, 2.25% Saline.
Horse.....	100 %	100 %	100 %	100 %
Beef......	6.25	6.25	12.5	3.13
Human ...	4.16	1.56		
Goat......	3.13	0.00		
Dog......	3.13	1.56	6.25	1.56
Sheep.....	2.0	0.00		.78
Pig.......	2.0	0.00	1.56	1.56

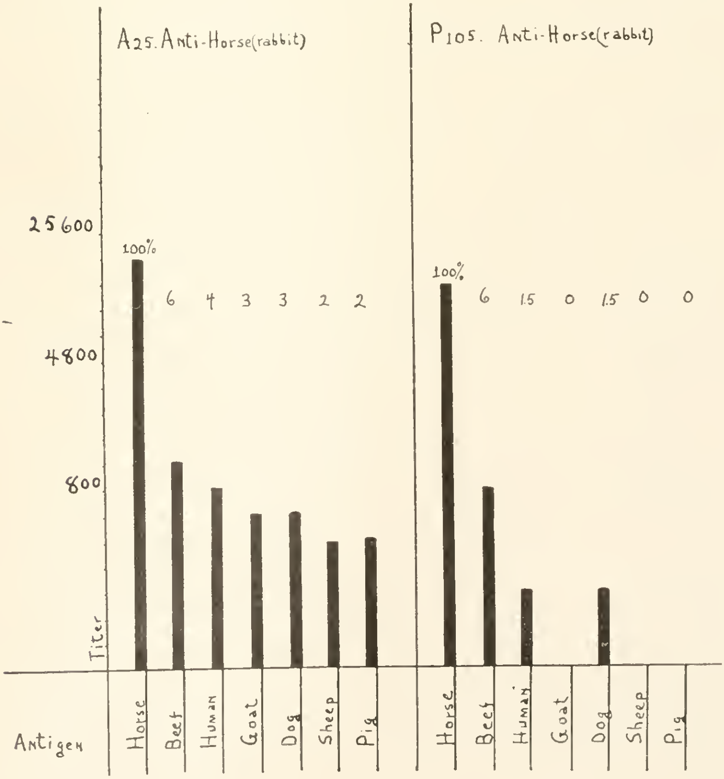

FIG. 8. Comparison of two anti-horse sera from rabbits.

same relationship to beef whether an antibeef serum was titrated with pig or whether an antipig serum was titrated with beef. It would seem then that this principle of reciprocal relationships could be used as a test of the truth of the values obtained in the precipitin reaction. Table XIII. gives the values obtained in these reciprocal titrations. The titrations which failed to exhibit correspondence are marked with an asterisk.

Table XIII.

Reciprocal Values of Antisera.

Antiserum.	Anti-gen.	Per Cent.	Antiserum.	Anti-gen.	Per Cent.
Rabbit sera			*Rabbit sera*		
A22 (antibeef)	Sheep	66	P40 Antisheep	Beef	75
	Pig	16.6	A21 Antipig	"	12.5
	Horse	16.6	A25 Antihorse	"	6.25*
	Dog	16.6	P140 Antihog	"	12.5
A24 (antibeef)	Sheep	50	P37 Antisheep	"	50
	Pig	8.3	A23 Antipig	"	12.5
	Horse	8.3	P105 Antihorse	"	6.25
	Dog	8.3	P123 Antidog	"	4.7
P37 (antisheep) . . .	Pig	8.3	A21 Antipig	Sheep	6.25
	Horse	4.16	A25 Antihorse	"	2.08
	Dog	4.16	P125 Antidog	"	2.4
P40 (antisheep) .	Pig	3.1	A23 Antipig	"	12.5 *
	Horse	3.1	P105 Antihorse	"	0.0 *
	Dog	3.1	P140 Antidog	"	12.5 *
A21 (antipig) . .	Horse	9.4	A25 Antihorse	Pig	2.08*
	Dog	4.7	P123 Antidog	"	.8 *
A23 (antipig) . .	Horse	6.25	P105 Antihorse	"	.00*
	Dog	4.6	P140 Antidog	"	9.4
P123 (antidog) .	Horse	2.4	A25 Antihorse	Dog	3.13
P140 (antidog) . .	Horse	12.5	P105 Antihorse	"	1.56*
Chicken sera			*Chicken sera*		
C8 (antibeef) . . .	Sheep	100	C11 Antisheep	Beef	75
	Pig	12.5	C4 Antipig	"	16.6
	Horse	6.25	C14 Antihorse	"	12.5
C11 (antisheep) .	Pig	0.0	C4 Antipig	Sheep	8.3 *
	Horse	8.3	C18 Antihorse	"	.78*
C4 (antipig) .	Horse	8.3	C14 Antihorse	Pig	1.56*

Twenty reciprocal values have been obtained with rabbit antisera and of these twelve gave correspondence within the limits of error. Of the eight which failed to give correspondence five involved horse serum and two involved dog serum. Of six chicken reciprocal titrations three gave correspondence and three failed to do so. Two of the latter involved horse serum. Clearly the horse serum was aberrant in its reactions. Nuttall reports similar results with horse serum. There may then be certain animals whose blood sera are not suitable for studies of this kind. If now only the beef, pig, sheep, and dog reciprocal titrations are considered, out of fifteen pairs of values eleven gave agreement, a rather high percentage.

The entire series of reactions recorded in these titrations seems to justify these conclusions:

1. That there is a variation in the response of different animals of the same species to the same protein.

2. That in spite of this variation there is a general agreement in the group reactions obtained with the majority of the antisera tested. This correspondence is independent of the homologous titers of the antisera compared (provided the antisera are sufficiently strong to react with all the proteins tested) and may be found even in antisera produced by such different animals as rabbits and fowls.

3. That in rabbit antisera there is an inhibition of response to other rodent bloods which is absent in fowl antisera.

4. That where there is a variation in the group reactions of two antisera of the same kind it is possible to tell which is the truer series of values by making reciprocal titrations. Only those values which check within the limits of error of the reaction may then be taken.

Discussion.

The building of phylogenetic trees has practically ceased today. This is not by any means because the problems of animal relationships are believed to be finally settled. It seems rather to be an expression of hopelessness as to the possibility of solving these problems. It is true that there is general agreement as to the relationships of animals within certain main branches of the animal kingdom. But there is no such agreement as to the affinities of many of the phyla of Invertebrates to each other or to Vertebrates. But even within these main groups of animals we have failed to reach agreement as to what constitutes a species or to learn how to express quantitatively the relations of one species or genus or family to another. For classification has been based chiefly on adult morphology, the tendency being to throw aside the evidence from developmental morphology as being more liable to error. However this may be, morphology itself is subject to certain limitations. Chief among these are two (1) an error due to convergence which it is difficult to estimate with certainty, (2) the failure to give a quantitative expression to morphological features in general and hence the necessity of depending much on interpretation as to what various structures may mean in descent. Interpretations differ with interpreters and hence it is that there is endless difference of opinion as to the relationships of certain groups of animals necessitating countless "revisions" of them. The discovery of an objective, quantitative test which could be applied to animals and through which some general agreement as to their relationships might be attained would be a great help to the zoölogist.

Attempts to study other than gross morphological structures of animals for the purpose of understanding their relationships have already been made. The study of the distribution of respiratory pigments and related substances is an instance in point. While it has been found that hemoglobin is rather erratically distributed among Invertebrates, the gaps may partly be filled with animals possessing related substances. The study of the crystallography of the hemoglobins by Reichert and Brown (1909) is another instance. The results obtained by these authors confirmed in general the existing classification. Chemical analyses of various tissues have also been made but such methods fail for the most part to show stereochemic differences between substances of essentially the same elementary constitutions. It seems to be true, however, that it is generally such stereochemic differences or resemblances which are most important in the study of related animals. We must have a method therefore which may identify and give some quantitative expression to these differences. Such a method seems available in the precipitin reaction.

Perhaps then the most important contribution to the study of animal relationships, aside from morphology, can be made through the use of the precipitin reaction. For the results obtained by it can be expressed in a quantitative manner, and are therefore entirely objective and independent of the interpretation of the observer. The use of the precipitin reaction must, however, be carefully controlled. Though this reaction has been shown to be very sensitive, nevertheless it is subject to a fifty to one hundred per cent. possible error as used in these experiments. Furthermore it is incapable of distinguishing between closely related animals. It is affected by the H-ion and salt concentrations, but most important of all *it must be standardized through the use of secondary antigen solutions of known protein content.* With these precautions the results obtained through its use by everyone will become strictly comparable and of considerable value.

From the data on relationships obtained with this limited series of animals it is felt that Nuttall's statement is essentially true. For the degree of reaction between an antiserum and various animal bloods has in general paralleled the relationships among those animals. Thus nearly always the reaction of a given antiserum is greater with animals belonging to the same order than with members of other orders. The only exception to this has been the horse serum (Perissodactyla) which sometimes was reacted upon more strongly or equally strongly by an antipig or an antibeef or an antisheep serum than were some other sera belonging to the order Artiodactyla. The relationship between Artiodactyla and Perissodactyla has always been considered to be close, the two usually being included in one order, the Ungulata. The precipitin reaction reveals this close relationship. The results on horse serum seemed irregular, however, and not too much emphasis is to be put upon them.

When essentially the same series of reactions may be obtained from various antisera of the same kind, whether they be produced in fowls or rabbits, the probability that these reactions correspond to some reality in the relationship of the animals concerned becomes very great. And when, furthermore, the reciprocal values obtained with two or more different antisera check within the range of error, as happened in the majority of these titrations, the probable correctness of this conclusion becomes still greater.

Much criticism of Nuttall's work has appeared in recent years in Europe. Statements have been made that the precipitin reaction did not follow at all the relationships of the proteins tested. The results given here do not support such views. Never has any heterologous reaction in this entire series exceeded the homologous reaction of the same antiserum, as Friedberger and Collier (1919) or Friedberger and Meissner (1923) claim. Perhaps the fault lies with their technique for they have apparently used neither buffered salt solutions nor standard antigens of known strength. Furthermore the dilutions which they made of antigen were only 1–100; 1–200; 1–1,000; 1–10,000; and 1–20,000. The possible error in their readings must therefore be 1,000 per cent. in most cases. If such crude methods had been used in these tests no doubt the results obtained would correspond to theirs but the value of the precipitin reaction in the study of relationships would have been entirely overlooked. A better technique must be used if results of value are to be obtained. It is not that poor results cannot be obtained with this reaction, the important thing is that good results can be obtained and poor results recognized as such and discarded from further consideration.

The fact that the values obtained in this study of a limited series of animals correspond closely with the results of Nuttall's quantitative tests shows that the precipitin reaction can give essential uniformity of results. Having thus proven its value in the study of Vertebrates where relationships are relatively certain, it may now be applied to the study of animals of more uncertain affinities. The Invertebrates offer a large and important field for such research. A few results have already been obtained by Nuttall in the study of Crustacea. Perhaps also his demonstration of the affinity of the King Crab (Limulus) to spiders is to be taken as an indication that the further application of the precipitin reaction to the study of relationships of Invertebrates will eventually yield results of some value to the phylogenist.

CONCLUSIONS.

1. The possible error in reading the ring test in this study has been 50–100 per cent.

2. The sensitivity of the ring test is very great.

3. The titer of an antiserum is directly proportional to the concentration of antigen within its limits of reaction.

4. Total nitrogen determinations, at least, should therefore be made on all antigens in order that comparable results may be obtained.

5. The specificity of the reaction of an antiserum decreases with time.

6. The H-ion concentration of antigen solutions affects the reaction. Therefore buffered salt solutions should be used.

7. Increase of salt concentration from .85 per cent. to 2.25 per cent. decreases the titer of chicken antisera and tends to increase their specificity.

8. Filtration of antisera through Berkfeld filters usually does not decrease titer nor change specificity.

9. There is a variation in response of different animals of the same species to the same protein.

10. There was a general agreement in the group reactions obtained in the majority of cases. This correspondence was independent of the strength of the antisera used and occurred in antisera produced in such different animals as rabbits and fowls.

11. There is an inhibition of the response of rabbit antisera to other rodent bloods which is absent in the fowl.

12. The principle of reciprocal relationships can be used to test the agreement of the values obtained.

13. The ring test is quantitatively specific for no heterologous reaction ever exceeded the homologous reactions of the same antiserum.

14. The ring test when properly performed may give information of value to the student of animal relationships.

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NOTES ON THE FORM AND FUNCTION OF THE GOLGI APPARATUS IN STRIATED MUSCLE.

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It is a curious, not to say remarkable, fact that among the hundreds of papers published since 1898 on the morphology and function of the Golgi apparatus and the mitochondria, very few indeed have been seriously concerned with these cytoplasmic elements as they presumably occur in striated muscle "cells." This undoubtedly means, not that the matter has been overlooked, but that it has been looked over and found very difficult of solution. The whole question of the structure of striated muscle being still in a somewhat uncertain state, it is not surprising that the morphology of its generalized cytoplasmic components should likewise be unsettled. Indeed this could be anticipated, since the extraordinary differentiation of muscle fibers may well involve morphological modifications of the Golgi apparatus and mitochondria of such a nature as to render uncertain their immediate identification. Still it is clear that an understanding of the morphological disposition of these cytoplasmic elements might throw much interesting light not only upon their own mode of functioning but upon that of muscle substance as well.

The tendency in recent years, upon such infrequent occasions as the matter has been mentioned, seems to have favored the identification of the so-called Cajal-Fusari network[1] with the customary Golgi apparatus of other tissues. The morphology of this network, first described by Cajal and later by Fusari, was subsequently studied in great detail by Veratti ('02),[2] whose paper is illustrated by a remarkable series of figures. Veratti found, in essential agreement with Cajal and Fusari, that there is present in the striated muscle fiber an elaborate system of very delicate fibers, which form an anastomosing network rather easily blackened in a specific manner by the so-called black reaction (reazione nera) of Golgi. This network consists primarily of a series of regularly arranged cross-fibers, located in various but definite planes as determined by the phenomena of cross-striation in the muscle fiber itself. These cross-threads are connected by more irregularly arranged fibers running parallel to the muscle columns. This whole network has been shown with reasonable certainty to occupy the interstices between the muscle fibrils, lying thus in the sarcoplasm but having an entirely independent existence. The general accuracy of these findings has subsequently been substantiated by Martinotti ('04), Sánchez ('07) and particularly Holmgren ('08).

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[1] It should be carefully noted that the terms "network" and particularly "reticular apparatus" have been frequently used in discussing this matter in striated muscle without any intention of implying that the material thus denominated is necessarily homologous with the true Golgi apparatus or network.

[2] This paper contains a very complete bibliographical review of the whole subject to which reference may be made for the papers of Cajal and Fusari. These were unfortunately published for the most part in journals not readily accessible to

It was shown, furthermore, especially by Veratti that the regular arrangement of the network is gradually arrived at by progressive differentiation of a network which is much less regularly arranged in the embryo, for example of mammals. Martinotti ('04) was able also to show that a return to a more or less embryonic condition of the network could be effected in the rabbit by starvation, or section of the fibers of the nerve supply. But in this direction Veratti went still further by demonstrating that the condition of the network at birth varies in different mammals. Thus in the mouse and rabbit, the final arrangement of the net is not achieved until some days after birth; while in the guinea-pig, the final (adult) condition is present at birth, and an arrangement comparable to that of the mouse at term, can be found only in the guinea-pig fetus about fifteen days before birth. In this connection also it is of interest to note that the results for instance of M. Sánchez ('16) on the Purkinje cells of rabbits, show that the Golgi apparatus in these cells only attains its adult development and arrangement by a gradual

most American workers, but a general idea of the results can be found in Cajal ('90) and Fusari ('94). It may also be noted here that the relation of the Cajal-Fusari network revealed by silver nitrate, to the network in striated muscle stained by gold chloride, is still a matter of doubt. In these notes, the latter network will not be considered.

evolution in the first weeks after birth. This at least suggests the possibility that in other nerve cells the adult arrangement of the Golgi material may likewise be a product of post-embryonic development—depending of course in detail upon the kind of cell and its time of functioning with respect to birth, etc.

I have digressed here into these details of the differentiation of the Golgi network because of their remarkable connection with the relations recently developed by Tilney, between the beginning of functional activity of a given muscle and the time of myelinization of the corresponding nerve tracts. It would appear that the nerve fiber is not the only part of the mechanism which at birth is still in an incomplete state, but that the muscle fiber and possibly also the nerve cell itself have still to perfect their organization subsequent to birth. Here the agreement in the guinea-pig between the completion of myelinization before birth as noted by Tilney and the perfection of the muscle network as noted by Veratti, with the known activity of the newborn young of this animal, seems to me of extraordinary interest. It thus appears that the failure of the nerve-muscle mechanism to operate immediately after birth may be due not only to the lack of the myelin sheaths but to the fact that the system as a whole is still in process of completion.

The possible relation of this muscle reticulum to the Golgi apparatus was first discussed by Veratti, who refrained from drawing any definite conclusion. Sánchez ('07), however, concluded that this network represents the apparatus of Golgi-Holmgren, and though he had in mind Holmgren's conception of a trophospongium, it is equally clear that he intended an homology with the Golgi apparatus as conceived by other workers. At about the same time Holmgren ('08) published an account of the network in many kinds of muscle cells, and endeavored to prove that it represented the trophospongium described by him in other kinds of tissue. Golgi himself seems to have accepted the homology of the muscle reticulum with his 'internal reticular apparatus.' In his review of 1911, Duesberg considered the case unproved, but in 1914 he states that "the hypothesis of Golgi is probably correct."[1] The only obvious criticism of this conclusion which occurs to me is that in insects the Golgi apparatus seems characteristically to occur in the form of scattered Golgi bodies, rather than a network comparable to the condition usually found in mammals. On the other hand, the silver methods have been tried so often, and by so many people, that if there is a Golgi apparatus in the insect muscle fiber other than the network mentioned, it ought by every possibility to have come to light. It may therefore be tentatively concluded that the so-called muscle "network of Cajal-Fusari" represents the Golgi apparatus (and all of it) in striated (skeletal and heart) muscle fibers of both vertebrates and invertebrates.

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[1] The not very convincing account and figures of Luna ('11) on mammalian heart muscle must thus be considered as probably incorrect.

It is to be noted that all the results thus far obtained have depended upon the use of silver nitrate, usually following the Golgi fixation in osmium-bichromate. It would be interesting to know whether other methods, which have proved specific for the Golgi apparatus, would reveal the same network, or material arranged in some new way. Recently while studying some preparations of Cowper's gland from the cat, I found some fragments of striated muscle which, inadvertently included with the glandular tissue, had been impregnated by osmic acid according to Nassonov's ('24) modification of Kolatchef's method. Following this treatment a great variety of pictures results. The cross striations may be very clearly demonstrated, after the well-known reaction of osmic acid on striated muscle. Various other ways of impregnating the muscle substance also occur, of which an interesting one is the specific blackening of the sarcoplasm while the muscle columns or fibrils are relatively unstained. A cross-section of a portion of a muscle fiber thus impregnated is shown in Fig. 1.

In one case a very unusual impregnation was found, in which both muscle fibrils proper and sarcoplasm failed to blacken in the least, while fragments of a network lying between the muscle columns were rather clearly impregnated. A small section of such a muscle fiber is shown in Fig. 2. The muscle columns are indistinctly differentiated as longitudinal bands. The network consists of longitudinal and transverse threads along which granular thickenings occur at rather regular intervals. Except for the fact that the longitudinal fibers are unusually numerous (but see Martinotti '04), these threads seem to correspond in a general way to the Cajal-Fusari reticulum, here revealed in a fragmentary manner.

Added proof is thus brought that this network, responding to the silver nitrate and osmic methods for impregnating Golgi material, actually is the homologue of the Golgi apparatus. It seems to me that the final demonstration of this homology can be obtained only by a study of the development of the network from a stage in which the original condition of the Golgi apparatus will permit its identification beyond dispute. Such an identification seems to have been made by Fañanás ('12), who claims that in the chick embryo (from the sixth day on) the Golgi apparatus of the muscle cells (tubular phase) can be made out as granules and rods localized in the protoplasmic axis of the fiber. Concerning the forms and metamorphosis undergone by this material until the complicated net of the mature muscle fiber is completed, he states that he will treat in detail in a special paper. But I can not find that this paper has appeared.

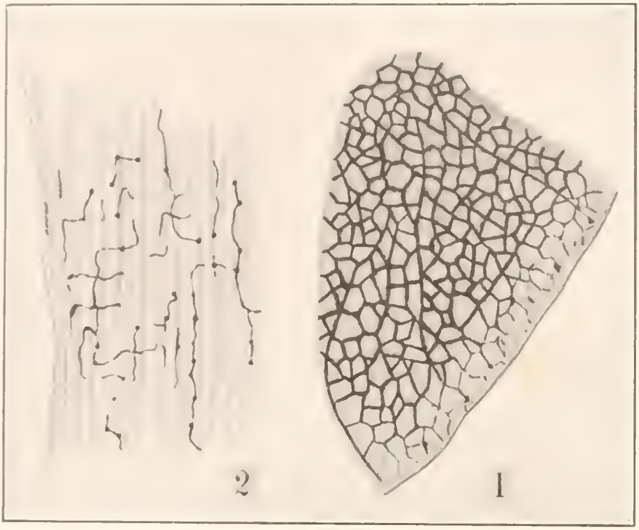

Fig. 1. Portion of a cross-section of a striated muscle fiber from the cat, showing the sarcoplasm impregnated with osmic acid; sarcolemma appears along the right-hand border. Fig. 2. Longitudinal section of the same kind of muscle, showing fragmentary impregnation by osmic acid of the muscle reticulum of Cajal-Fusari (= Golgi apparatus?). Both figures × 2150.

Assuming that the Cajal-Fusari reticulum actually is the Golgi apparatus of a striated muscle fiber, there is raised at once the interesting question of the functional relation of this material to the functional activity of the muscle itself. It was thought by Cajal that the network, in the insect muscle upon which he worked, was in one sense or another a continuation of the tracheal network. This view was supported by Sánchez ('07), and elaborately developed by Holmgren ('08), who found in the network the trophospongium of his familiar hypothesis. According to Holmgren, in insects this network is in reality a protoplasmic extension of tracheal end cells located on the surface of the muscle fibers; while in vertebrates (and other forms where tracheal tubes are lacking) it is a similar extension of materials essentially to be related to the sarcolemma, or "trophocytes" connected with it. To most workers this would probably seem a very perilous expedient for saving the homologous nature of these networks, especially in view of the fact that the trophospongium in many kinds of cells has been shown to be the Golgi apparatus pure and simple, and quite lacking in its supposed relationship to external trophic cells. As a matter of fact, the supposed relation of the network to the tracheal tubes in insect muscle fibers was investigated and discussed by Veratti, who denied the connection described by Cajal (and later Holmgren). To me the evidence seems to indicate that the networks are essentially independent of extra-muscular formations, but that possibly in insects, tracheal endings may be closely associated with the fibers of the network in a purely spatial sense. While therefore rejecting Holmgren's theory, it is nevertheless interesting to note further that he found in this network an area of metabolic transformations comparable to that of the trophospongium in other cells.

Bearing in mind these facts of morphology and supposed functional significance, it has proved interesting to me to consider the problem in the light of the recent development of opinion on the rôle of the Golgi apparatus in cells generally. From this it begins to appear that the Golgi apparatus is very generally involved in the synthetic operations leading to the formation of various secretory products, including many extracellular enzymes. When, therefore, we find an apparent homologue of the Golgi apparatus in such extraordinary relations to the contractile material of muscle fibers, the suggestion comes to us at once that the functional significance of the Golgi apparatus may here be of the same general character as in at least some other kinds of cells. Thus we might look upon the Golgi apparatus in muscle cells as the possible seat of oxidase formation. Such possibilities are not, however, within the range of cytological examination at present, and some more tangible suggestion will be essential if the matter is to be carried further.

It happens that a very large mass of evidence has already been accumulated, especially through the work of Holmgren ('08, '10, '13, etc.), concerning the positional relationship of the 'Golgi apparatus' in muscle fibers to the so-called Q- and J-granules (or sarcosomes), as well as the remarkable behavior of these bodies during the cycle of muscle action. It has been demonstrated, particularly by Holmgren ('10), that these granules undergo most remarkable changes in staining capacity coincident with the contraction and relaxation wave. These changes, furthermore, are of such a character as to indicate that they are probably related to a transfer of material between granules and muscle columns, presumably necessary to muscular activity. In addition, Holmgren ('10 and '13), Knoche ('09) and others, following up the discoveries of earlier workers, have shown that these granules present many remarkable similarities in staining behavior to the so-called half-moon bodies long since described by Heidenhain in the pelvic gland cells of salamanders. There is here the distinct suggestion that the sarcosomes (*i.e.*, the Q- and J-granules) are actually a specialized type of secretory granule, related primarily to the Golgi network of the muscle fiber, and undergoing a well determined cycle of exhaustion and replenishing synchronous with the contraction and rest of the adjacent muscle columns.

It is not my intention to enter here into a detailed development of these speculations, for which Holmgren's papers suggest many interesting possibilities. Such speculations are hardly warranted as yet, especially because of the conflicting data concerning the distribution of the mitochondria in striated muscle fibers. This matter has in fact received no very extensive examination, but several workers, and their opinion is concurred in by Duesberg ('10 and '11), have identified the Q- and J-granules as the mitochondrial constituents of striated muscle. But the staining behavior of these bodies changes rapidly in a manner not characteristic of chondriosomes and the evidence that they are of such a nature seems to depend on what might be termed an argument from logical necessity, viz., these bodies sometimes stain like chondriosomes and they have some appearance of being the only granules available which could be so identified. As a matter of fact, a figure of heart muscle given by the Lewises ('24, Plate VI., Fig. b), and Duesberg's ('10) Fig. 24 of the voluntary muscle of the fowl, seem to indicate that the mitochondria are really not the Q- or J-granules at all, but granules with a quite different disposition. The matter evidently is one to be analyzed further, using some basis of identification other than that of staining reactions.

In conclusion, therefore, my purpose in this short résumé has been to bring out our uncertainty concerning some very essential structures in striated muscle fibers; at the same time to indicate the probable disposition of the Golgi apparatus, and to point out various possibilities which this disposition at once suggests. We have now to reëxamine the whole problem in order to establish the homology of the network with the Golgi material of embryonic cells; and to determine the true significance of the Q- and J-granules—whether chondriosomes, possible secretory granules or some as yet unrecognized combination of the two. The solution of these problems should throw much interesting light on the functional significance of the Golgi apparatus, and perhaps also of the mitochondria.

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THE SPERMATOGENESIS OF *UMBRA LIMI* WITH SPECIAL REFERENCE TO THE BEHAVIOR OF THE SPERMATOGONIAL CHROMOSOMES AND THE FIRST MATURATION DIVISION.

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Introduction.

Recent advances in the genetics of the Teleosts have given an impetus to the study of the chromosomes of fishes. The discovery of an XX, XY type of sex-linked inheritance in *Lebistes reticulatus* (Schmidt, '20; Winge, '22), *Aplocheilus latipes* (Aida, '21) and *Platypœcilus* (species?) as studied by the writer,[1] indicating an XX, XO type of sex-linked inheritance, all point to the presence of heterochromosomes in the fishes, although none have as yet been recorded. Winge ('22) suggests the possibility of such elements existing in *Lebistes* but gives no cytological evidence. Geiser ('24) says in discussing the subject in *Gambusia*, "If one were to trust to the uncertain help of analogy, it might be claimed that *Gambusia* has one heterochromosome." Agar ('11) shows in the heterotypic division of *Lepidosiren paradoxa* (although he does not indicate them as such), two large chromosomes, almost identical as to size and shape. These chromosomes show a tendency to behave like the sex-elements in those forms in which they have been definitely worked out. The nature of these chromosomes and their similarity of size and shape would seem to indicate, if they are sex-chromosomes, that in *Lepidosiren* the male is homozygous for sex and should show the poultry type of sex-linked inheritance.

Upon reviewing the literature on the spermatogenesis of the Teleosts, it becomes apparent that there are many features in the germinal cycle of these forms which are not clear, and that these should be studied further before proceeding with the problem of the sex-chromosomes. During the summer of 1923 a great number of different kinds of fish were collected (bass, perch, blue-gills, sunfish, stickleback, pickerel, carp, and mud-minnows) and their testes preserved, sectioned, and examined. After careful examination the mudminnow (*Umbra limi*) was selected as the most favorable specimen for study. *Umbra* offers especially good material as the spermatogonial cells are large, with the chromosomes in the form of L's and V's. They also show no great tendency to clump, hence individual chromosomes and chromosome pairs may be picked out with a fair degree of certainty. The first maturation division is especially clear, showing definite meiotic stages and clean separation of the tetravalent chromosomes at the metaphase so that, in the selection of this form, the writer believes that he has avoided the main difficulties encountered by most investigators on fish spermatogenesis, such as: (1) The small size of the germ-cells, especially the primary and secondary spermatocytes; (2) the small size of the chromosomes together with the fact that they usually are in the form of threads, rods, or dots, rendering counting extremely difficult and making it practically impossible to follow them through the various stages in spermatogenesis; and (3) the tendency of the chromosomes to clump badly.

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[1] Data as yet unpublished.

The purpose of the present paper is to try to clear up some of the little known or little understood phases in the spermatogenesis of the bony fishes. The writer wishes to express his appreciation of the aid of Dr. A. S. Pearse in collecting specimens and in reading the manuscript, of Dr. L. J. Cole for pointing out the genetic application of the problem, and of Dr. M. F. Guyer for suggestions in technique and helpful criticisms on the entire work.

Materials and Methods.

The fishes used in this investigation were collected in the vicinity of Madison, from Lake Mendota and from various small streams which empty into Lake Mendota and Lake Wingra. Collections of *Umbra limi* were made once a week and testicular material was fixed every day, or every other day during the time the cells in the testes were undergoing proliferation. The testes are two elongated white bodies situated in the posterior part of the body-cavity just ventral to the swim-bladder. They are oval in shape tapering from the middle toward each end. The testes do not fuse as they do in many fish but remain separate throughout their entire length. It was found that material from fish which were kept in the laboratory for more than a week could not be relied upon, as it began to show abnormal behavior.

For cytological study material was fixed in Carnoy's, Gilson's and Flemming's strong fluids, Flemming's modified according to Hance ('17), and Bouin's fluid modified according to Allen ('16). Allen's modification of Bouin's fluid was found to give the best results, although Hance's modified Flemming gave fair fixation. The testes were dissected out and quickly immersed in the fixative, and then cut into very small pieces. The fixative was kept at the same temperature as the water of the aquarium which contained the fish. Safranin and gentian violet, or safranin counterstained with licht-grün or iron-alum hæmatoxylin counterstained with eosin, acid fuchsin, picric acid, licht grün, or Congo red were employed as stains. The best results with counterstains following Bouin's fixation were secured with solutions of licht grün and Congo red in 95 per cent. alcohol. Sections were cut from three to seven micra in thickness. Those of seven micra were best for studying the chromosomes and for making chromosomal counts in the large spermatogonial cells. Smears were also made but did not prove as valuable as the sections in this study.

SEASONAL CYCLE IN THE SPERMARY OF *Umbra.*

The testes of *Umbra* are usually full of spermatozoa up to the middle of May. Though there is some variation among individuals and in different years on account of differences in seasonal succession, expulsion of the spermatozoa usually occurs during the last week or ten days of May. Following this there is a period of rest in the testes until about the middle of July. The primordial germ cells or primary spermatogonia, as I shall designate them in this paper, are arranged around the periphery of the lobule before and just following the expulsion of the spermatozoa. This peripheral position of the primary spermatogonia in those lobules in which a few spermatozoa remain, give such lobules a superficial resemblance to the seminiferous tubules of the higher vertebrates. Turner ('19) describes a similar method of peripheral arrangement in the pickerel. There is very little if any division of the primary spermatogonia during this period in the testes. Each new spermatogonium is formed by a process of growth from a migrating cell; once established, it eventually becomes the center of a cyst within the lobule. This process of cyst or pocket formation begins at the periphery and gradually extends toward the center of the lobule until the latter is filled with a solid cord of germ-cells. Following the formation of this primary cord of cells, other small migrating cells may be seen along the periphery of the lobule or between the cyst walls during the period of migration and growth which reaches its apex by the middle of July. Up to this time few mitotic figures can be seen. From the middle to the last of July there is a period of rapid multiplication of the primary spermatogonia and in a number of the cysts the cells have advanced as far as the first maturation division. By the first week in August the cells in a number of cysts have completed maturation and show various stages in the transformation of the spermatids. During the remainder of August and early September the various maturation stages are to be found in abundance. By the middle of September a large proportion of the testes are filled with spermatozoa. With the completion of the transformation of the spermatids into spermatozoa all evidence of cyst arrangement within the lobule disappears. Just before and immediately after expulsion of the spermatozoa, which occurs during the following season, there is no evidence of cysts within the lobules. They are formed again at the beginning of the next migration and multiplication period.

There is no antero-posterior seriation in the testis of *Umbra;* each cyst completes its cycle as an independent unit. A cyst in which late primary spermatocyte figures can be seen, however, may also show earlier maturation divisions and some spermatogonia. Likewise secondary spermatocytes and transforming spermatids may be found in the same cyst. This makes detection of the different stages in the spermatogenetic cycle more difficult. Another factor which complicates the study is the seasonal variation which influences, to some extent, the seasonal cycle in the testis. During the summer of 1924, a comparatively wet and cold season, testicular cysts which during a normal season would show a great many maturation divisions had not advanced any further than the formation of spermatogonia.

SPERMATOGONIA.

1. *Origin.*—How is the new crop of germ-cells formed at the beginning of each seasonal cycle? Are they produced from reserve germ-cells which in turn are descended from other reserve cells, or are they formed anew from migrating cells which come from some point outside the lobule? Turner ('19), found the latter to be true for the perch. Hargitt ('24), working on the adult salamander (*Diemyctylus viridescens*), found that the spermatogonia are formed anew each season from the epithelial cells which line the collecting tubules or from the peritoneal tissue which covers the testis. Although no effort has been made by the writer to trace the origin of the early spermatogonia outside the testis, there seems to be little doubt that in *Umbra*, as in the perch, the primary germ-cells or early spermatogonia arise anew each season from cells which migrate into the lobules from some outside point. A certain amount of migration and growth, indeed, seems to be in progress throughout the entire cycle of spermatogenesis. Occasional small ameboid cells are to be seen along the lobule wall or between the walls in small numbers during August and early September when the testes are filled with spermatogonia, spermatocytes or spermatids and spermatozoa. In material collected from the first to the middle of September, during which time most of the testis becomes filled with spermatozoa, single large resting cells or clusters of cells may be found scattered throughout the lobules. Whether these degenerate or remain in a resting state with an occasional division is not known. Clusters of large cells are found in isolated cases still persisting in the fall but since no such cysts are found the following spring, it is inferred that they have given rise to spermatozoa during the winter. The large isolated cells which may be found along with a few ameboid cells in lobules which have completed their cycle of spermatogenesis may

remain over as suggested, or they may degenerate. No positive evidence can be presented on this point although similar large cells, showing occasional mitosis, can be observed throughout the time that the testes are filled with spermatozoa.

The origin of the early spermatogonia can be best observed just before the liberation of the spermatozoa. At this time the new germ-cells, which are to be found around the periphery of the lobule, are of varying sizes, ranging from small migrating cells (Fig. 1) to very large cells (Fig. 2) in a growing or resting condition. Of twenty-four linear measurements taken at random, cells were found to range from 19.50 to 58.50 micra. The migrating cells (Fig. 1) are ameboid in shape and have a darkly staining nucleus with a thin investing sheath of cytoplasm closely applied to the nuclear membrane. The nuclei take the stains more deeply than the nuclei of the large resting cells in the same lobule. The migrating cells (Fig. 1) are oblong or flattened with a prominent chromatin nucleolus suspended in the nuclear reticulum near the nuclear membrane. The chromatin granules are quite large and peripherally arranged around the nuclear membrane.

2. *Growth of the Spermatogonia.*—As soon as the migrating germ cells become lodged at the periphery of the lobule the growth period of the spermatogonia begins. This growth period is of special interest in *Umbra* and other Teleosts for in these forms the great increase in cell size during spermatogenesis is thrown back into the early spermatogonial stages. In most animals the growth period occurs in the primary spermatocyte stage and it is usual to find the spermatogonia of almost equal size. The precocious growth period of Teleosts may be due to a number of factors: (1) All the spermatogonia formed during any one season are ultimately used up. (2) The method of cyst-formation in the lobule, in which each migrated germ-cell becomes the primary center of a cyst; since each seasonal crop of germ-cells arises anew from migrating cells. (3) The long period of rest in the testis following its depletion of spermatozoa, during which time there is an active reorganization of the testis itself conditioned by the constant arrival of new germ-cells from some point outside the lobule. (4) The shortness of the time between

the close of spermatogonial multiplication and the first maturation division. Certainly if there is any increase in size in the primary spermatocyte in such forms as *Umbra* it is very slight as compared with the increase found in the primary spermatocytes of most animals. The spermatogonial growth period is accompanied by very little mitotic activity of the cell. It is initiated at the time the migrating cells assume a resting position in the lobule and persists up to the first week in August or thereabouts, although small clusters of isolated cells in a growing condition may, as already indicated, be found throughout the entire cycle of spermatogenesis. The large cells of the spermatogonial growth period and the cells resulting from their first division are the most favorable for chromosome study. Such cells (Fig. 2) in a resting condition may show one to three chromatin nucleoli. The chromatin granules are more centrally arranged than in the migrating cells (Fig. 1) and scattered along the linin network. The reticulum (Fig. 2) appears more compact than in the early migrating cells. These early growing or resting cells show (Fig. 2) from eighteen to twenty-one small compact bodies which react deeply to nuclear stains and are scattered throughout the reticulum. Their relative constancy in number and the fact that they correspond very closely to the number of chromosomes found in the spermatogonial cells, suggests the possibility of their being prochromosomes. They appear in the early resting or growing stages of the cell as deeply staining bodies scattered throughout the reticulum. In a very early prophase, as the chromatin granules begin to collect along the linin threads, these bodies seem to be the centers (Fig. 3) toward which the migrations of the chromatin granules progress. A somewhat later prophase shows the chromatin aggregated along the linin threads in a beaded condition. Although larger chromatin centers may still be seen, the beaded structure (Fig. 4) of the forming spireme in the middle prophase, makes further identification of these bodies as possible prochromosomes uncertain. The evidence goes to show, however, that these chromatin bodies are relatively constant as to number and are aggregation centers for the future chromosomes. No cells were observed in which such bodies could be seen in the reconstruction period following the telophase in the dividing spermatogonia. All traces of the chromatin nucleoli, as such, have disappeared by the time of a mid-spireme formation. In the late spireme the beaded appearance of the thread disappears although its outlines remain rough up to and including the early prophase chromosomes. The spireme breaks up into definite chromosomes, twenty-two in number.

By the time the spermatogonial growth period has reached its height a great number of degenerating spermatogonia may be seen throughout the testis. These degenerating spermatogonia, and later, degenerating early spermatocytes, may be found in lobules in considerable numbers up until the time the lobule becomes filled with spermatozoa, but not later.

The prophase chromosomes of the spermatogonial divisions in *Umbra* are very long and thread like, and irregular in outline. No definite equatorial plate seems to be formed. Agar ('11) found a similar condition to exist in the spermatogonial cells of *Lepidosiren*. Subsequent metaphases or late prophases of these chromosomes show marked variation as to length, shape, thickness, and regularity of outline. Fig. 5 shows an early metaphase stage in which the chromosomes are all long and curved and mainly in the shapes of V's and L's; they clearly display differences in size, thickness, and shape. Still earlier, however, no such marked condition is to be seen, for the chromosomes are then all approximately of the same size, length, and thickness.

Figures 5 to 13 and 56 to 60 show progressive condensation changes in the chromosomes. These series of figures show that a gradual contraction takes place in all the chromosomes until a condition is reached like that shown in Figs. 12 and 13. Agar ('12), working on *Lepidosiren paradoxa*, found a similar condensation taking place in its chromosomes, the chromosomes contracting toward the apices of the V's. Figs. 12 and 13 show spermatogonial cells which exhibit this condition to a marked degree. Such contraction phases can be easily demonstrated in early spermatogonial cells because in this growth period such cells are very large, and the multiplications which take place are very slow. Later and smaller spermatogonial cells, in the more active multiplication period, do not show such conspicuous gradations, although they do show the chromosomes to be in the shape of V's and L's. The writer believes that the condition as shown in Figs. 12 and 13 is unusual. Such figures are found in small numbers and usually occur at the periphery of a lobule or cyst and in small isolated clusters in the ends of the testis. The origin of the chromosomes in the form of V's and L's seems to be the more usual occurrence. The more unusual type of chromosomes have been of service in this study, however, since they furnish valuable material for checking chromosome counts made in other cells. Where the chromosomes appear as they do in Figs. 5 to 11, counting is difficult because they are not all in one plane. Some lie above the others, often in as many as three planes, and such an arrangement makes an accurate count often very difficult.

The chromosomes in the large spermatogonial cells are so clear that it is not difficult to pick out the homologous pairs. With this purpose in mind the chromosomes of Figs. 6 to 10, have been listed in Figs. 56 to 60 according to size. It will be noticed in all of these figures that although the chromosomes vary somewhat in shape at different times, two of them, labeled (*a*) in Figs. 6 to 10 and Figs. 56 to 60, although differing in size in the different cells have a fairly constant and characteristic shape. The members of this pair may be found side by side but more commonly one or two other chromosomes intervene. The fate of the chromatin nucleolus has not been fully determined in this study. Other investigators have shown that certain so-called nucleoli really constitute the sex chromosomes. Painter ('24), says: "It is concluded that the chromatin nucleolus is made up of the sex chromosome element in the opossum and presumably in all other mammals." The nuclei of the spermatogonial growth period and those at the end of the multiplication period (Figs. 15 to 17) in *Umbra* may show chromatin nucleoli varying from one to three. One is of more constant occurrence (Fig. 15), however, and it is in only a minor number of instances that two nucleoli are found in those spermatogonial cells which go into the maturation divisions. The relative constancy in size and shape of the two chromosomes just referred to, in all the spermatogonial cells studied, points to the possibility of their being sex-chromosomes, although no more positive evidence than this can be presented at this time. If they are sex-chromosomes the inference is that they would bring about the poultry type of sex-linked inheritance in *Umbra;* in other words the male would be of the two X, the female of the single X type. The condition here seems to be much the same as that depicted by Agar ('11), in *Lepidosiren;* according to his figures 18 to 31 two large chromosomes almost identical as to size and shape can be followed through the maturation division of the first spermatocyte, although he does not indicate that they are sex-chromosomes.

3. *Multiplication.*—Multiplication takes place to a small extent throughout the spermatogonial growth period. The new cells produced by these divisions grow somewhat and probably take part in the formation of some of the cysts within the lobule. If the first divisions of the early migrant cells are considered as the beginning of the multiplication period, then this period is a prolonged one. About the middle of July, however, active multiplication of the cells in some cysts is in progress. Such multiplication in *Umbra* is very rapid, once the reorganization of the lobules of the testis has taken place. Practically all of the lobules have completed their reorganization by the beginning of the first week of August. As before mentioned this reorganization of the lobules into cysts takes place not only through an enlargement of the migrating cells but also to some extent through their multiplication. Though isolated cysts in a lobule may still show cells in the spermatogonial growth-phase, the great majority of the cysts within any one lobule have practically or entirely completed their multiplication and are ready for the first maturation division by August. On the other hand a goodly number of cysts within the lobule have, by that time, completed the entire cycle of spermatogenesis. Turner ('19) says of the perch, "As each spermatogonium gives rise to a group of descendents they form a cyst and all pass through the same stages of division at the same time"; and this is in the main also true of *Umbra*. If one considers a cyst in *Umbra* as the product of a single spermatogonium, then an estimate of the number of spermatogonial divisions which have occurred in any one cyst

may be arrived at by counting the number of spermatogonia in a cyst which is ready for the first maturation division. On such a basis there are at least six generations.

The cells of the late multiplication period are usually so small and the chromosomes so massed that counts of the chromosomes are not satisfactory. In so far as counting can be done the numbers check with those obtained in the earlier cells where counts were possible. Although twenty-two chromosomes may be observed it is practically impossible to identify homologous chromosomes in these cells.

Turner ('19) describes in the perch a nuclear contraction of the transforming spermatogonia accompanied by the extrusion of liquids or particles into the cytoplasm. In *Umbra* no such phenomena occur. In all spermatogonial mitoses a definite centrosome appears at each pole of the spindle. The early spermatogonia of the spermatogonial growth period (Fig. 2) are from three to six times as large as the resultant spermatogonial cells of the last multiplication (Figs. 15 to 17) division. For example; the average early growth-cells range in diameter from 31.20 micra to 66.30 micra, while late spermatogonial cells which are ready for the first maturation division, have an average diameter of about 19.50 micra. While there are minor exceptions, the majority of the cells in the different cysts are approximately of the same size just before the first maturation division. This and the succeeding divisions are very rapid in *Umbra*. This is probably true in most of the Teleosts, and may be the reason why so many investigators have found difficulty in finding the various stages in fish spermatogenesis. It is necessary to cut and examine a very large number of sections in order to find the proper stages. In this study over five hundred slides were made and examined.

THE FIRST MATURATION DIVISION.

1. *Growth of the Primary Spermatocyte.*—At the time of the transformation of the spermatogonia of the last multiplication division (Figs. 15 to 17) into primary spermatocytes the spermatogonial cells may show one to three deeply staining chromatin nucleoli. The nucleoli apparently separate into chromatin granules.

During the transformation of such spermatogonia into spermatocytes they undergo an increase of 15 to 20 per cent. in size by the time they have attained the late pachytene stage.

2. *Leptonema, Pachynema and Zygonema.*—At the beginning of maturation, the chromatin nucleoli seen in the spermatogonia disappear. The leptotene threads are formed by a condensation of the chromatin granules on the linin network of the nucleus. The nucleus becomes filled with a mass of fine intertwining threads (Figs. 18 and 19) of which it is impossible to determine the exact number. From this step the succeeding stages of presynizesis follow each other in rapid succession. Fig. 20, an early zygonema, shows some irregular pairing of the threads. Figs. 21 and 22, judging from the increasing proportion of thick strands, show further advancement in the pairing of these threads. Fig. 23 represents a late zygonema or early pachynema in which the fusion has been completed in the mid regions of the threads, but in which the ends still remain single. Fig. 24 shows a stage, the pachynema, in which the fusion has been completed and a definite spireme is formed containing approximately one half the number of threads of chromatin found in preceeding stages. Although a definite count of these threads has not been attempted, by comparing this stage with the previous leptotene stage one can readily see it displays fewer threads.

Synizesis and Diakenesis.—Soon after the formation of the spireme of pachytene threads, the latter begin to concentrate toward the periphery of the nucleus (Fig. 25), leaving the center clear, with only a few chromatin strands connecting the peripheral masses. This massing of the pachytene loops around the nuclear membrane marks the beginning of synizesis (Fig. 25). There seems to be a definite tendency for the chromatin material to concentrate (Fig. 26) into one or the other of two areas at opposite sides of the nucleus, thus indicating a marked polarity. Fig. 27 shows a still more advanced stage of synizesis in which the chromatin is collected in the form of loops, principally toward one pole of the nucleus. The chromatin strands at the opposite pole are concentrated and do not show the looped condition. Two loops or rings (Fig. 27) can commonly be identified in this stage at one side of the two main chromatin masses near the nuclear membrane. These two rings are connected by a thin strand of chromatin material. They apparently lose their identity in the following stage. Fig. 28 shows a further contraction phase. In this stage the chromatin masses at each pole draw away from the nuclear membrane and collect toward one side of the nucleus. Fig. 28 shows the remaining portion of the smaller mass being incorporated into the main mass. This marks the end of the period of synizesis.

Immediately following synizesis the nuclear membrane disappears. Fig. 29 shows the unravelling or breaking up of the chromatin knot into bars and smaller masses of chromatin. At this point eleven irregular chromatin bodies corresponding to the reduced number of chromosomes can be recognized. Figs. 30 and 31 show a further unravelling of these knotted masses or bars of chromatin. Rings or loops are to be seen appearing from the denser masses as shown in Fig. 29. These stages (Figs. 29 and 30) mark the final passing of the stage of synizesis.

The rings of chromatin which are to be seen in late diakenesis appear to be derived in two ways from the synizesic mass: (1) Long threads of chromatin segment after the unravelling of the previous stage and may be seen in twos (Fig. 61—*n* and *o*) one wrapped around the other, having the appearance of a definite strepsinema which might permit of a "double or triple cross over." Typically these threads apparently unwrap, however, and later condense into rings, each pair constituting such a ring. (2) Shorter segments may be seen in early stages following synizesis which arrive at the ring stage by a sort of unravelling or pulling apart as shown in Figs. 32 and 33, and (*a*) and (*b*) of Fig. 61. This pulling apart appears to take place at the ends first and later, a space appears between the two threads at the middle (Fig. 61—*c*, *d*, *e*, *f*); the ends having come into contact again.

Figure 61 (*a*) to (*p*) shows various diakenetic figures in the contraction and formation of the rings; these may have been derived from either condition previously described. Figs. 31 to 35 show various stages in the formation and further contraction of these rings. In Fig. 34 several chromatin figures in the form of Y's are to be seen. These may have been produced either by the cutting across of some of the previously described loops, or they may be due to a further contraction of the loops in which the bivalents at one end of the Y have entirely united while the other end still shows the split which may be observed in the various diakenetic figures previously cited. The double split indicating the tetrad nature of these chromosomes, is not evident until they come upon the metaphase spindle of the first maturation division where end views can be secured (Fig. 36). At this stage, however, the tetravalent nature of the chromosomes is readily observable after proper staining and destaining methods. No irregular behavior, such as lagging or irregular divisions (Fig. 37) in early or late anaphase, was seen in these chromosomes. They pull apart forming dyads (Figs. 39 to 41), eleven in number in each daughter cell or secondary spermatocyte.

The Second Maturation Division.

The secondary spermatocytes (Fig. 38) are about one-third smaller than the spermatogonia which entered the first maturation division. Although resting nuclei (Fig. 38) are formed not infrequently, in secondary spermatocytes, the more common method of procedure is for the telophase of the primary spermatocyte to become rearranged immediately into the metaphase of the secondary spermatocyte. Both Geiser ('24) working on *Gambusia*, and Agar ('11) on *Lepidosiren* found likewise that no resting stage occurs between the telophase of the primary and the metaphase of the secondary spermatocyte. The writer believes that the condition found depends mainly upon the rapidity with which cell-division is going on. When the rate is slow a resting stage will be found but when rapid it is dispensed with. In *Umbra* the transition period between primary and secondary spermatocytes as well as the time of the secondary spermatocyte division itself is usually very brief. Figs. 39 to 41 show such a rearranged metaphase in which the chromosomes retain their definite bivalent condition. They still show their dyad nature plainly when at the equator (Figs. 39 to 41) of the spindle for the metaphase of this division. At a somewhat later time (Figs. 42 and 43) they tend to fuse or adhere to one another so closely that they cannot be counted. The late metaphase or early anaphase spindles (Fig. 43) viewed from the side exhibit no unusual behavior of the chromosomes. No odd or lagging chromosomes are to be seen. The dyads are halved or pulled apart apparently along the longitudinal split indicated in the tetrads of the previous primary spermatocyte, and eleven small univalent chromosomes go to each daughter cell or spermatid. Fig. 44 shows a late anaphase in which chromosomes may be seen in a univalent condition.

Nuclear Changes Occurring During Transformation of the Spermatid.

The resting spermatids are approximately one half the size of the secondary spermatocytes. Only the nuclear behavior of the transforming spermatid will be considered. No attempt has been made to work out in detail the part taken by the cytoplasm or centrosomes in the process. A prolonged period ensues between the resting phase of the spermatid (Fig. 45) and the mature spermatozoan. At the beginning of spermiogenesis the chromatin collects along the linin of the nucleus in a rather scattered condition in which roughly seven to eleven chromatin masses (Fig. 46) may be distinguished. At the same time this initial condensation of the chromatin masses on the linin network of the cell is occurring, the axial filament begins to form. The tail, developed to about its full length, is present shortly thereafter. As the chromatin masses appear on the linin of the nucleus, their behavior is indicative of the formation of chromosomes for the metaphase of another division but before this process goes far the chromatin begins to migrate toward the periphery of the nucleus (Figs. 46 and 47). At this stage, scattered around the nuclear membrane, may be seen roughly five dense chromatin masses (Figs. 47 and 48), about twice as large as those which were visible in the more diffuse condition. These chromatin masses, up to and throughout this phase, remain connected by lighter staining strands with the linin of the nucleus. The appearance is that of a fusion of the smaller chromatin bodies of the previous stage in pairs. Following this condition there is a dense massing of the chromatin material around the nuclear membrane (Figs. 49 and 50), leaving the center of the nucleus clear with only a few linin fibers connecting the masses at the periphery. Immediately following this peripheral arrangement of the chromatin in the form of a dense anastomosed ring (Figs. 50 and 51) beneath the nuclear membrane and around the clear center to the nucleus, a contraction of the nucleus takes place. Figs. 52 and 53 show final stages in the arrangement of the nuclear material to form the head of the spermatozoon. The heads of the spermatozoa formed by this contraction are somewhat smaller than the original spermatid nucleus. The heads of the mature spermatozoa are practically spherical (Fig. 55) although some may show such shapes as the one pictured in Fig. 54.

Discussion.

The literature on the spermatogenesis of the fishes or fishlike vertebrates is very limited. There is apparently no detailed piece of work relating to the Teleosts.

The prevalent opinion on the origin of the germ-cells in spermatogenesis is that they are formed either from cells which migrate into the testes, assume a fixed position, and produce the ultimate crop of spermatozoa, or that they are derived from reserve germ-cells which have existed as such since the formation of the first or primordial germ-cells in early embryogenesis. Moore ('95), working on the spermatogenesis of several genera and species of Elasmobranchs, Rawitz ('99), working on Scyllium canicula, Cunningham ('86), ('91), in two papers on the spermatogenesis of the Cyclostome (Myxine glutinosa), presuppose the origin of the primary spermatogonia from a germinal epithelium. Upon the spermatogenesis of the fishes proper, there are the papers of Agar ('11), ('12), on the spermatogenesis of *Lepidosiren paradoxa*; Turner ('19), on the seasonal cycle in the spermary of the perch; Geiser ('24), on the spermatogenesis of *Gambusia holbrooki*; and Winge ('22), on the cytology of Lebistes reticulatus. Winge does not discuss the spermatogonia, Agar assumes a development from a definite germinal epithelium, while Turner and Geiser assume an origin from migrating cells. Turner ('19) shows that in the perch the cysts are formed anew each season and attributes the formation of the early spermatogonia to cells which migrate into the testes from a cord of germ cells outside. Geiser ('24) says: "The definite sex-cells by repeated divisions give rise to the final spermatogonia . . . . The cysts of spermatogonia are the result of the continued fission of the germ-cells which at the season of sexual activity migrate to the periphery of the gonad." A recent article on the origin of the germ-cells in the adult salamander is of special interest here. Hargitt ('24), working on the salamander (*Diemyctylus viridescens*) shows that the cysts do not retain residual spermatogonia, but the new crop of spermatogonia is formed each season from migrating cells of the peritoneal covering of the testis or from the epithelial lining of the collecting ducts. Hargitt found isolated nests of germ-cells in the mesenteries and peritoneum outside the testis but he does not attribute the origin of the new spermatogonia in old cysts to these cells. From his observations on the behavior of the germ-cells in the testis of *Umbra* the writer is led to believe that, as in the perch (Turner, '19), and in the salamander (Hargitt, '24), the primary spermatogonia are formed anew each season from cells which migrate from some point outside the lobules and possibly outside the testis. No cords of germ-cells were found outside the testis of *Umbra*, however, as described for the perch by Turner. The evidence points more to some such type of origin as that described by Hargitt for the salamander.

I find the seasonal cycle in *Umbra* to coincide in the main with that found in the perch by Turner ('19). Two distinct differences might be pointed out: (1) Beginning with the multiplication period the spermatogenetic cycle in *Umbra* is more rapid than that found in the perch. (2) The writer believes the migration of the new germ-cells to be a continuous process in *Umbra* though slower at some times than at others, instead of being restricted to any one particular period as was found in the perch.

The growth phase of the primary spermatogonia varies in different fishes. Turner ('19) working on the perch, and Geiser ('24), working on the top-minnow (*Gambusia*), found a definite growth period. In the perch the primary spermatogonia are about three times the size of the final spermatogonia and in *Gambusia*, eight times the size of the final spermatogonia. It was found in *Umbra* that they are from three to six times the size of the final spermatogonia. Agar ('11) differentiates between primary and secondary spermatogonia in the Dipnoan, Lepidosiren. He finds that there is a decrease in nuclear size from thirty micra to seventeen micra during the transition from primary to secondary spermatogonia.

In the Dipnoi the final spermatogonia are only about one third the size of the primary spermatocytes. In this respect the Dipnoi appear to resemble the Amphibia more than they do the Teleostomi. It would be interesting to know what the condition is in the Elasmobranchs and Cyclostomes. The writer believes that this early growth phenomenon in the early spermatogonia in *Umbra* and other teleosts is bound up with the seasonal cycle in these forms; namely, the formation of spermatozoa from all the spermatogonia, the absence of reserve germ-cells, the destruction of the cysts of the lobule at the end of spermatogenesis together with their reformation the following season, and the formation of the early spermatogonia from migrating cells.

The number of generations these early spermatogonia go through before the final spermatogonia are produced varies in different fishes. Turner ('19) finds, in the perch, that there are five or six generations, Geiser ('24), in *Gambusia* ten to twelve. In *Umbra*, at least six generations are passed through before the final spermatogonia are formed.

Chromosome numbers have been given for the following species:

	Male.	Female.
Perca flavascens (Turner, '19)	27	
Gambusia holbrooki (Geiser, '24)	35 or 36	
Lebistes reticulatus (Winge, '22)	46	46
Lepidosiren paradoxa (Agar, '11)	38	
Scyllium canicula (Moore, '95)	24	
Scyllium canicula (Rawitz, '99)	20 to 24	
Umbra limi	22	

The behavior of the spermatogonial chromosomes in the fishes has been stressed little. Agar ('11) ('12) goes into the matter in Lepidosiren in some detail. He finds, as I do, that the chromosomes of the newly formed equatorial plates are long and in the shape of L's and V's. Many of these chromosomes go through a contraction at the apices of the V's until small transversely constricted chromosomes are formed. Discussing the contraction phenomenon, Agar ('12), says: "It becomes operative especially, but not solely, whenever the chromosomes are short in comparison with their length, as happens normally in meiosis and exceptionally in somatic tissues." I find in *Umbra*, as has already been stated, numbers of spermatogonial cells showing varying degrees of contraction; this contraction is apparently toward the apices of the V's, as Agar has already pointed out. Early metaphase or late prophase (Fig. 5) in *Umbra* may occasionally show very long chromosomes with little or no evidences of contraction. Different cells show late metaphases and early anaphases in varying degrees of contraction. Anaphases may be found, although quite rarely, with chromosomes similar to those represented in Fig. 14. A more contracted state than that shown in Fig. 5, is probably the normal condition arrived at in metaphase. Extreme contraction, as shown in Figs. 12 to 14, are found in medium sized cells embedded between larger cells, in the regions of the testes where most connective tissue abounds, and at the periphery of the lobules. This extreme contraction is, I believe, the unusual rather than the usual occurrence. This condition as well as those that have been previously described, I interpret as being due to relative growth rates of the cells. Cells dividing rapidly do not show contraction to any appreciable extent while those dividing more slowly and remaining in each phase of mitosis longer, exhibit it more markedly. The slow growth in the extremely contracted phase may be due to tension exerted upon such cells by the connective tissue surrounding them, or by the larger and more actively growing neighbor cells. Contraction, however, is to a certain degree a regular procedure, and the shorter chromosomes, as was found by Agar in *Lepidosiren*, contract first and most. In *Umbra*, however, contrary to the view of Agar, I am inclined to believe that these transversely constricted chromosomes play no considerable part in meiosis.

Some investigators have reported a definite growth period, others no such period, in the primary spermatocyte divisions of the fishes. In the Dipnoans and Amphibia the primary spermatocytes are three times the size of the spermatogonia of the last multiplication division. Turner ('19) finds that a growth period does not occur in the perch. Geiser ('24) shows (Figs. 11 to 21) a definite increase in size in the primary spermatocytes of *Gambusia*. In *Umbra* there is unquestionably a slight increase in nuclear size over that of the preceeding stage. It is probably due to the fact that the primary spermatocyte stage is one in which the cells remain longer than they do in the multiplication or secondary spermatocyte phases. All spermatocyte stages may be equally rapid in the perch, and this may perhaps be the reason Turner found no increase in nuclear size.

Maturation has been infrequently studied in the fishes or the Elasmobranchs. Moore ('95) has given a very good account of the essential points in the maturation divisions of Scyllium canicula, considering the technique employed by the workers of his time. The method of reduction described differs in no essential manner from that recognized in late years as the orderly procedure in most animals and plants. After the conjugation of the leptotene threads and formation of a synaptic knot (synizesis) the chromosomes segment into long thick rods of chromatin which pair and form a closed ring type of tetrad. The subsequent separation of the parts of the tetrad is accomplished by a transverse split through the center of the loop. The second division is similar to the first, with the exception that there is no reduction, namely, the separation of the dyads by a transverse split. The dyads separate and twelve univalent chromosomes are distributed to each daughter cell or spermatid. It is generally believed now that what Moore describes as a "split" in the ring is the pulling apart of homologous chromosomes at their previous line of fusion. Rawitz ('99) working on *Scyllium* states that at the time of reduction there is no reduction of the chromatin number, but that there is a reduction of the chromatin material by disintegration. He finds fourteen to sixteen chromosomes in the primary spermatocyte and fewer and larger chromosomes in the secondary spermatocyte. It may be that Rawitz has confused the spermatogonial and spermatocyte divisions. Geiser ('24) finds that in the early stages of diakenesis the tetrads are of the open ring type, later becoming closed, at which time all evidence of their tetrad nature is lost. In *Umbra* during diakenesis, the chromatin segments into blocks of varying thickness which first pair up and form open rings, but later contract and form closed ones (Fig. 61). A further contraction at the time of the metaphase shows these tetrads condensed until all appearance of their tetrad nature has disappeared. In fact, unless carefully scrutinized, they may be mistaken for dyads. Upon destaining, however, it can be readily seen that they are arranged at the metaphase as typical tetrads (Figs. 35 and 36). Fig. 37 shows such tetrads arranged on the spindle for the first maturation division. The condition in *Umbra* is very similar to that shown in Figs. 45 to 48 by Moore ('95) in *Scyllium canicula*. In *Umbra*, as in *Scyllium*, the fiber attachment is median (Fig. 37), and the tetrads separate into dyads, the separation occurring along a longitudinal axis similar in manner to the opening of two V's which have been placed in apposition to each other. Separation begins at the apex of the V's and ends at the basal region. Thus dyads are formed which go to the secondary spermatocytes.

The behavior of the chromosomes in the secondary spermatocytes is not as easily followed as in the primary because of the greater irregularity of the chromosomes in lining up at the metaphase and of their subsequent merging together (Fig. 42) soon after they assume definite form. Furthermore, the chromosomes are much smaller than in the preceding division. In very early metaphase (Figs. 39 to 41), however, they can be readily made out as dyads and can be seen to pull apart (Fig. 43), eleven univalent chromosomes going to each spermatid. Another factor complicating the study of the chromosomes of the secondary spermatocytes is their proneness to rearrange immediately into the metaphase of the secondary spermatocyte from the telophase of the primary spermatocyte.

Winge's ('22) Figs. 8*a* to 8*c*, on diakenesis in the oöcyte of *Lebistes*, indicates a method of tetrad formation similar to that found in *Scyllium*, *Gambusia*, and *Umbra*. Agar ('11) found in *Lepidosiren* that at the conclusion of synizesis the chromosomes appear in the form of rings which later separate at their ends forming univalent chromosomes. Each such univalent member shortens and becomes transversely constricted. After dissolution of the nuclear membrane, according to him, the chromosomes unite at one end to form a long bar shaped (quadripartite) bivalent which bends on itself and forms a ring. In the metaphase of the first meiotic division one member of each bivalent goes to one daughter cell, the other to the other. If his observation is correct, this is an unusual type of reduction and is the only case reported for the fishes. In *Umbra* the reduction phenomena follow more closely the account given for *Scyllium* by Moore ('95). Although transversely constricted chromosomes do appear in *Umbra*, as described by Agar for *Lepidosiren*, they unquestionably occur in spermatogonial divisions in *Umbra* and never during the time of meiosis.

SUMMARY.

1. The spermatogonia of *Umbra limi* are formed anew each season from cells which migrate from some point outside the lobule, possibly in some cases even from outside the testis.

2. Evidence points to the presence of definite pro-chromosomes in the early spermatogonia.

3. The somatic and spermatogonial number of chromosomes is twenty-two. Chromosomes, at different times and in different cells, may exhibit contractions of varying degrees. Extreme contraction simulating a transverse fission is unusual.

4. Two large L-shaped chromosomes, relatively constant in size and shape, are found in all spermatogonial cells. They may be sex-chromosomes. If so, the male of *Umbra* is of the XX type.

5. The multiplication divisions of the spermatogonia are in general very rapid, with the result that the final spermatogonia are only of from one sixth to one third the volume of the original ones.

6. The primary spermatocytes of *Umbra* show roughly an increase in nuclear size of 15 to 20 per cent. over the spermatogonia of the preceding multiplication division.

7. The chromosomes emerge from synizesis in the form of rods or blocks of chromatin of varying length and thickness.

8. During diakenesis tetrads of the closed ring type appear. Final tetrad-formation is accomplished by the contraction of such rings. Carefully stained preparations show clearly the quadripartite nature of the tetrads in advanced metaphase when viewed from one pole.

9. Transversely constricted chromosomes are found in *Umbra*, but they are limited to certain spermatogonial divisions and play no part in the maturation divisions.

10. The tetrads have a median fiber attachment and separate into dyads at the metaphase of the first maturation division by a pulling apart at the apices of the loops or V's.

11. The secondary spermatocytes may go into a resting stage ibut usually the telophase of the primary spermatocyte passes mmediately into the metaphase of the second maturation division. The separation of the dyads at the second maturation division is very rapid.

12. No unusually large or lagging chromosomes were observed in the first or second maturation divisions.

13. The nuclear material of the transforming spermatid goes through an apparent fusion of chromatin masses, with a subsequent peripheral condensation of the chromatin, and a contraction of the entire nucleus as the head of the spermatozoön forms.

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EXPLANATION OF PLATES.

Unless otherwise indicated the figures on plates 1 to 3 represent a magnification of about 2160 diameters as they are reproduced. Figs. 7, 14, and 35 represent a magnification of about 1650 diameters. A camera lucida was used for drawing together with a 2 mm. oil immersion (Zeiss) and a No. 18 ocular (Zeiss). Drawings 51, 54, and 55, are from smears; the remainder, from sections.

Plate I.

Fig. 1. Ameboid cell of the migration period.

Fig. 2. Resting cell of the spermatogonial growth period showing a prominent chromatin nucleolus and twenty-one deeply staining chromatin bodies.

Fig. 3. Spermatogonium showing seventeen chromatin bodies which appear to be aggregation centers for the chromatin granules.

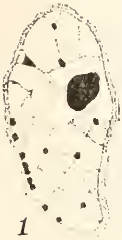
Fig. 4. Middle spireme of spermatogonial prophase showing the chromatin of the forming spireme in a beaded condition.

Fig. 5. Polar view of spermatogonial metaphase showing nineteen long and slightly contracted chromosomes.

Figs. 6, 7, 8, 9, 10, and 11. Polar views of spermatogonial metaphase showing twenty-two chromosomes in varying degrees of contraction. Probable sex chromosomes indicated at (*a*) in figures. Fig. 7 magnified 1650 diameters.

Figs. 12 and 13. Spermatogonial metaphase showing twenty-two transversely constricted chromosomes.

Fig. 14. Early anaphase of spermatogonium. Magnification 1650 diameters.



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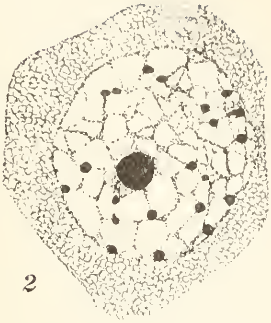

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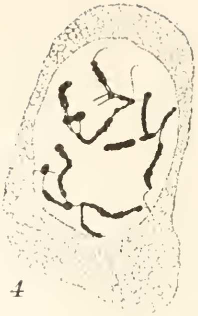

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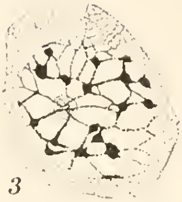

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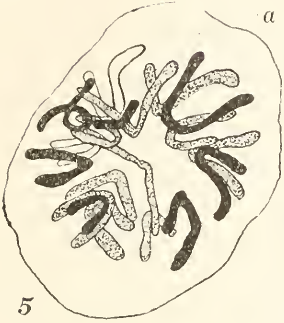

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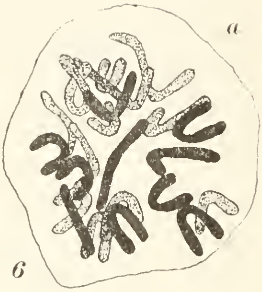

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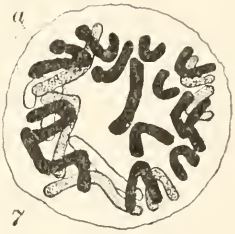

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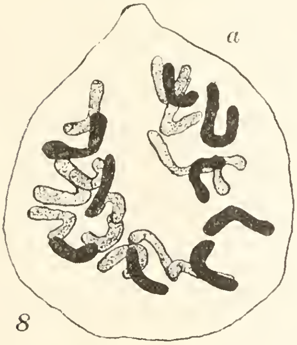

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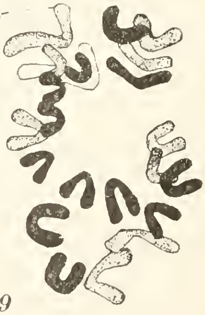

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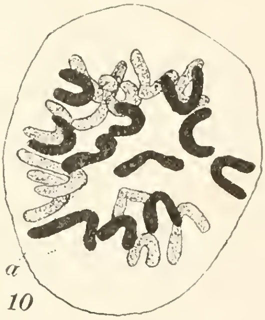

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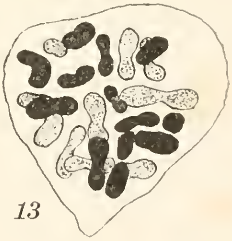

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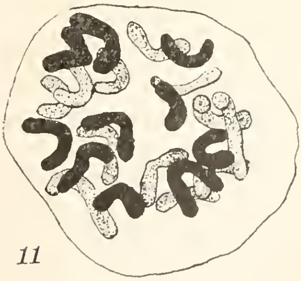

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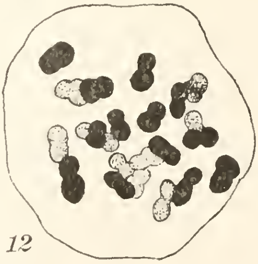

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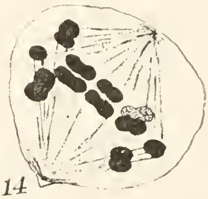

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PLATE II.

FIGS. 15, 16, AND 17. Resting spermatogonia of last multiplication division.

FIGS. 18 AND 19. Leptotene stages.

FIGS. 20, 21, AND 22. Zygotene stages.

FIG. 23. Late zygonema or early pachynema.

FIG. 24. Pachytene stage.

FIG. 25. Beginning of synizesis. The pachytene thread is massing around the nuclear membrane.

FIG. 26. Synizesis further advanced.

FIG. 27. Synizesis showing a looped condition of the chromatin.

FIG. 28. Synizesis completed. Complete contraction.

FIG. 29. Initial breaking up of synizesis. Only eight of the eleven chromatin masses are shown in figure.

FIG. 30. Final breaking up of synizesis.

FIG. 31. Continuation of unravelling process as shown in figure 30 and the beginning of diakenesis.

FIGS. 32, 33, AND 34. Various stages in diakenesis and contraction of the rings.

FIGS. 35 AND 36. Metaphases of primary spermatocytes showing tetrad nature of the chromosomes. Fig. 35 magnified 1650 diameters.

FIG. 37. Early anaphase of primary spermatocyte.

FIG. 38. Secondary spermatocyte in resting condition.

FIGS. 39, 40, AND 41. Early metaphase of secondary spermatocytes. Only six of the chromosomes are shown in figure 41.

FIG. 42. Late metaphase of secondary spermatocyte.

FIG. 43. Late metaphase spindle of secondary spermatocyte.

FIG. 44. Anaphase of secondary spermatocyte showing eleven univalent chromosomes.

FIG. 45. Resting spermatid.

FIGS. 46 TO 55. Various stages in nuclear behavior during the transformation of the spermatid. Figs. 51, 54, and 55 are taken from smears. Condition shown in figure 51 is similar to that shown in figure 52 but viewed from a different angle.

PLATE II.

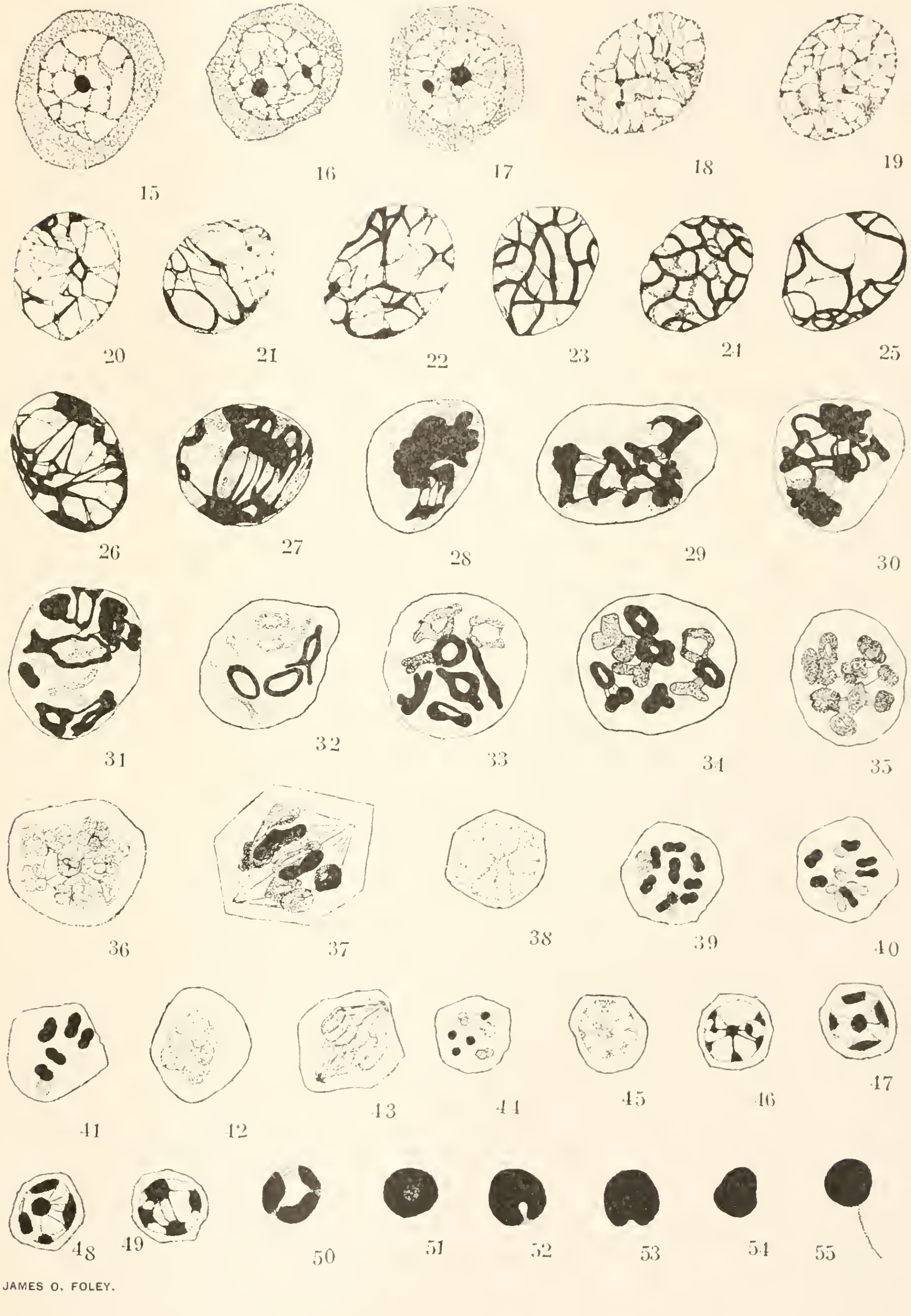
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Plate III.

Fig. 56. The individual chromosomes of Fig. 6.

Fig. 57. The individual chromosomes of Fig. 7.

Fig. 58. The individual chromosomes of Fig. 8.

Fig. 59. The individual chromosomes of Fig. 9.

Fig. 60. The individual chromosomes of Fig. 10.

Fig. 61. Various diakenetic figures selected at random from different primary spermatocytes.

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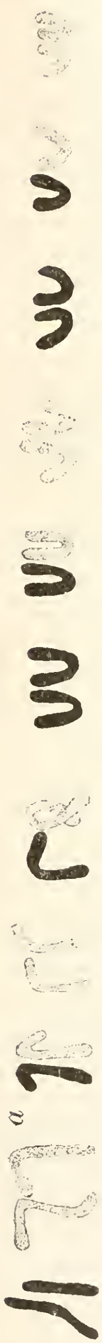

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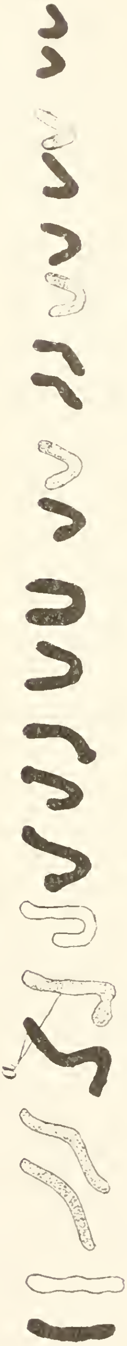

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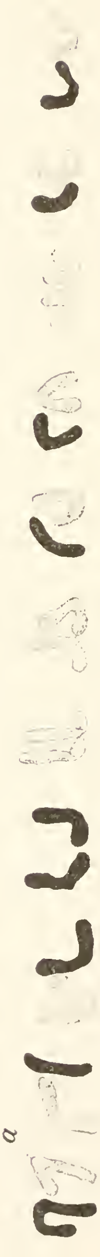

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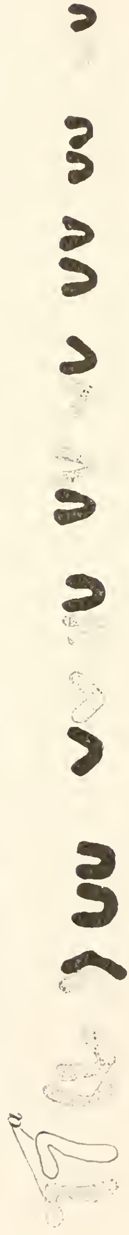

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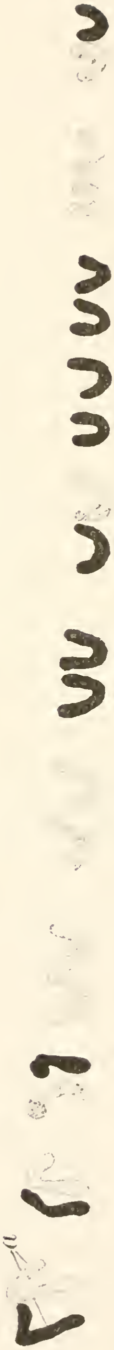

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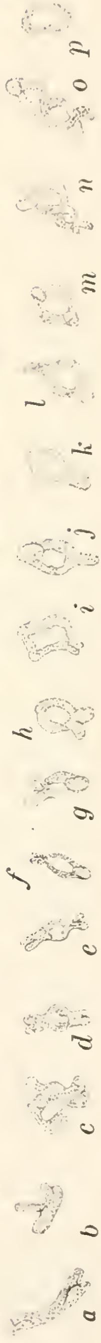
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A STUDY OF THE GENETIC RELATIONSHIPS OF THE "AMEBOCYTES WITH SPHERULES" IN *ARBACIA*.

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The pioneer work of Geddes (1880) marks the beginning of the study of the cellular elements in the perivisceral fluid of echinoids by means of modern methods. He was the first to recognize the different types of cells, and his classification has been the basis of all subsequent studies. In recent papers I have reviewed the work of those who have studied the reactions of these cells in the living condition both in the Echinoidea and Echinoderms in general (Kindred, '21 and '24). In both of these papers my own investigations have been limited to the functional reactions of the leucocytes, with only incidental remarks as to the "amebocytes with spherules." From my observations I have concluded that the leucocytes are the most generalized cells of the perivisceral fluid and that they are highly phagocytic, thrombogenic and scleroblastic. The "amebocytes with spherules" apparently carry on none of these functions. To be sure, they cling to the clot when it is formed by the thrombogenic leucocytes, but their part is passive. Various investigators have sought to find out where the amebocytes with spherules arise and what they do. Thus far no unequivocal evidence has been presented regarding function, nor have specific places of origin been recorded for these cells.

Attempts to locate the source of origin of the amebocytes with spherules have led to the applications of blood stains to smears and sections, with a resulting classification of the cells by their tinctorial reactions. St. Hilaire (1897) was one of the first to use these stains successfully and he classifies the cellular elements of the perivisceral fluid of the echinoids by their respective tinctorial reactions as follows: *a*, small leucocytes with neutrophilic granules; *b*, larger leucocytes devoid of granules, but having one or more nuclei; *c*, amebocytes with basophilic spherules; *d*, amebocytes with acidophilic spherules; and *e*, amebocytes with green spherules. Kollmann (1908), using the triacid stain of Ehrlich, following fixation with Zenker's fluid, does not find leucocytes with neutrophilic granules. Of the amebocytes with spherules, he studied only those with basophilic spherules. He states that these spherules are amphophilic. On comparing the tinctorial reactions of the several types of cells with their characters in the living condition, St. Hilaire interprets the basophilic spherules as identical with the colorless spherules of the living cell, the acidophilic as identical with the reds, and the green spherules as degenerate reds.

According to Kollmann the chemical composition of the colorless spherules is complex. They contain an albuminoid substance insoluble in alcohol and an alcohol-soluble lecithin. The red spherules seem to be of a more complex albuminoid nature and also contain a lecithin. The albuminoid nature of the spherules of those cells with colorless spherules accounts for their relatively slow rate of movement and also for their greater number in the connective tissue as contrasted with their number in the perivisceral fluid. Since the mast cells of vertebrates exhibit comparable tinctorial reactions and connective tissue affinities, Kollmann infers that the amebocytes with colorless spherules are comparable with them.

Kollmann also states that although he can find no relation between leucocytes and amebocytes with spherules, it is possible that the latter arise from the former by the development of spherules within the hyaline cytoplasm.

The following investigation has been undertaken to determine a possible source of origin for the amebocytes with spherules from leucocytes. The inspiration for this investigation came from the suggestion by Cuenot ('91) that the spherules are extrinsic in origin, nutritive in nature, and that the amebocytes with spherules may arise from leucocytes which have ingested food droplets.

MATERIAL AND METHODS.

Examinations were made of fresh drops and fixed smears of perivisceral fluid of Arbacia punctatum. Some of the smears were fixed in Helly's fluid and stained either with the Giemsa stain or with Ehrlich's triacid blood stain; others were fixed and stained by Wright's method; and those of a third group were fixed in the fumes of 2 per cent. osmic acid. By far the best and most uniform results were obtained with the Helly-triacid preparations. In addition to the smears, parts of the intestine were fixed in Helly's fluid, embedded in paraffin, sectioned at 7 microns, and stained with Ehrlich's triacid stain or with eosin-azure II. The eosin azure gave the most satisfactory results.

OBSERVATIONS.

Fresh Perivisceral Fluid.—The cellular elements present in the fresh perivisceral fluid of Arbacia have already been described in detail (Kindred, '21), hence a brief description of these cells will suffice here. The leucocytes are small, have a granular endoplasm containing clear vacuoles (acid to neutral red), and bear varying numbers of flap-like pseudopodia (Fig. 1). These cells have been observed to be phagocytic, thrombogenic and scleroblastic.

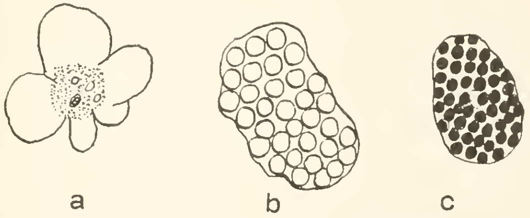

FIG. 1. Cells from fresh perivisceral fluid. *a*, leucocyte; *b*, amebocyte with colorless spherules; *c*, amebocyte with red spherules. × 1200.

Amebocytes containing either colorless, red, or yellow spherules are present in all drops of perivisceral fluid (Fig. 1). The amebocytes with red spherules are the most numerous, those with colorless spherules next, and those with yellow spherules least numerous. None of these cells have been observed to be phagocytic, thrombogenic, or scleroblastic. They move by means of blunt pseudopodia. These cells possess distinct cell membranes. The colorless spherules are larger than either the red or yellow spherules which are approximately of the same size. The nucleus of an amebocyte appears as a light area near the center of the cell.

Fixed and Stained Smears of Perivisceral Fluid.—In smears fixed with Helly's fluid and stained with Ehrlich's triacid stain, a very clear differential staining of certain cells occurs. The leucocytes are only slightly affected by the stain. No distinct granulations are present in the cytoplasm. The nucleus is, relatively large as compared with the cell body (Fig. 2).

The spherules of the amebocytes with colorless spherules are amphophilic to basophilic and stain violet to blue. Furthermore, these spherules do not retain their spherical shape (Fig. 2). The nucleus of this type of cell is small and compact as compared with the size of the cell, but it is actually as large as the nucleus of the leucocyte.

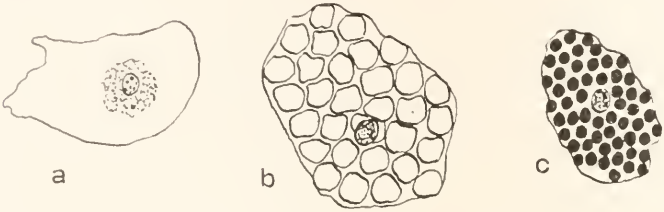

FIG. 2. Cells from smear of perivisceral fluid stained with Ehrlich's triacid stain. *a*, leucocyte; *b*, amebocyte with basophilic spherules; *c*, amebocyte with acidophilic spherules. × 1200.

The red spherules are acidophilic in reaction and stain a brilliant red (Fig. 2). These spherules retain their spherical shape, and are as sharply distinct in the fixed, as in the living material. Some of these spherules exhibit stages of degeneration and stain less strongly than others, a fact which leads to the inference that they may possibly be the spherules of amebocytes with yellow spherules, since the latter present no other definite tinctorial reaction. The relative number of cells with faintly acidophilic spherules corresponds in general with the number of amebocytes with yellow spherules in the living condition.

In smears fixed in the fumes of 2 per cent. osmic acid the spherules of the amebocytes are preserved and are sharply outlined in black. The color differences between the spherules are lost. The positive reaction of the spherule membrane to the osmic acid as contrasted to the solubility of spherule content is interpreted to mean that the albuminous part of the spherule is its content, and that the lecithin part forms the membrane.

Sections of the Alimentary Canal.—Transverse sections of the wall of the esophagus show a lining epithelium of tall and extremely narrow cells. This epithelium extends nearly across the whole thickness of the wall. Below the epithelium is a thin layer of connective tissue, reticular in character. This layer is invested by a thin tunica muscularis, superjacent to which is a delicate peritoneal epithelium. A similar relationship of layers exists in the wall of the intestine proper (Fig. 3), but the connective tissue layer is much thicker and thrown into folds which are followed by the epithelium. At the base of each connective tissue fold there are several lacunæ. In the meshes of the connective tissue strands there are numerous minute greenish droplets. From the positional relationship of these droplets to the intestinal epithelium, I infer that they are food droplets. From the extreme minuteness of the droplets nearest the basement membrane it seems that they have passed through the

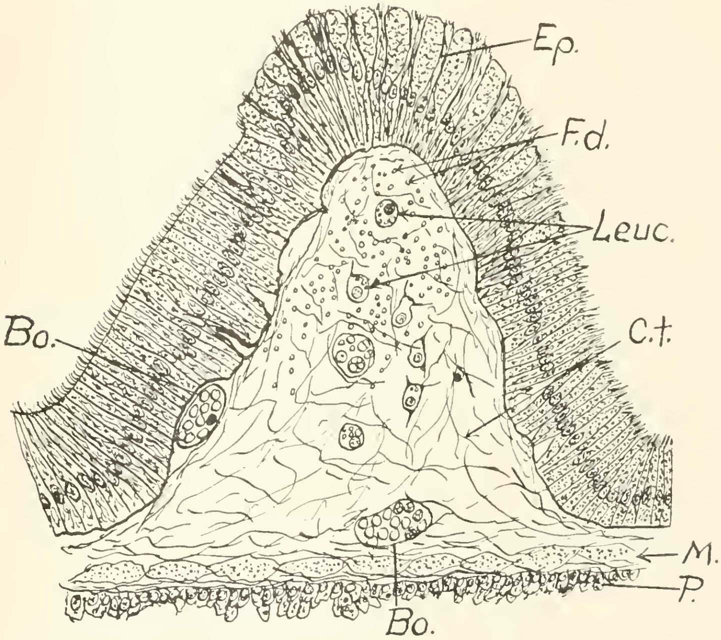

FIG. 3. Transverse section through part of intestinal wall. *Bo.*, amebocyte with basophilic spherules; *C.t.*, connective tissue layer; *E.p.*, epithelium; *F.d.*, food droplets; *Leuc.*, leucocytes; *M.*, muscular layer; *P.*, peritoneum. × 750.

cell membrane and basement membrane in fluid form not as droplets. Having reached the connective tissue the fluid is dispersed in the form of droplets.

Among the food droplets large numbers of leucocytes can be seen. Most of these leucocytes are in the act of ingesting droplets. The ingested droplets vary in number and in size (Fig. 3). In those cells which have ingested large numbers of droplets the cell body is distended far beyond the normal size of a leucocyte. The cells containing the most droplets are, as a rule, nearer the peritoneal surface of the intestinal wall. During the first stages of ingestion the droplets are smaller than the spherules of the amebocytes, but during the later stages of ingestion, the small droplets coalesce to form larger ones. The color of the droplets all through the first stages of ingestion is greenish and the droplets are apparently unaffected by the stain. As the droplets enlarge they acquire a slight basophilic reaction.

Cells with typical basophilic spherules are very numerous in the lacunæ at the bases of the regions of food ingestion and sometimes are found in the connective tissue meshes among the ingesting leucocytes. Amebocytes with acidophilic spherules, on the other hand, are seldom present in the intestinal wall.

Conclusions.

One phase of this investigation has demonstrated that the tinctorial reactions of the cells of the perivisceral fluid of *Arbacia punctatum* are identical with those of other echinoids. That is to say, the cytoplasm of the leucocytes is not tinctorially active, the spherules of the amebocytes with colorless spherules are basophilic in reaction, and the spherules of those with red or yellow spherules are acidophilic in reaction.

The second phase of this investigation reveals what I infer to be a genetic relationship between the leucocytes and the amebocytes with spherules. This inference depends primarily upon the interpretation placed upon the tinctorial reactions of the food droplets ingested by leucocytes in the intestinal wall. My preparations show leucocytes ingesting food droplets. It is also known that the leucocytes contain vacuoles giving an acid reaction to neutral red (Kindred, '21). Hence those cells in which the droplets begin to exhibit a basophilic reaction are probably those in which the acid content of the vacuoles has begun to act upon the droplets. Further action of this acid or digestive enzyme renders the cell one in which the droplets, now in the form of larger spherules, are distinctly basophilic in reaction. Thus amebocytes with colorless spherules are inferred to arise from leucocytes which have ingested food. Further evidence for this inference is given by the relatively great numbers of amebocytes with basophilic spherules in the intestinal wall in the region of food ingestion.

It is further possible to conceive that the colorless spherules undergo a gradual digestive change as the amebocyte containing them passes through the tissues of the body. Judging from the sequence of events in intracellular digestion in the Protozoa this change would be in the direction of acid to alkaline. Hence it is inferred that these colorless spherules, during the process of intracellular digestion, gradually give off part of their substance to the tissues through which the amebocyte containing them passes. Subsequently the spherules shrink in size and become alkaline in nature. This change in size and chemical nature could possibly be interpreted as the manner in which red spherules arise. The end product of the red spherules would be the yellows.

If my inference as to the genetic relationship between the amebocytes with colorless and red spherules is correct then the greater number of amebocytes with red spherules in the perivisceral fluid is caused by the accumulation here, near several avenues of escape, of degenerating cells. The idea that that the amebocytes with red spherules are degenerating cells is further borne out by observations which have shown that they pass out through the gills, or else they undergo crystalline degeneration in the test (List, '97).

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A COMPARISON OF MITOSIS IN CHICK TISSUE CULTURES AND IN SECTIONED EMBRYOS.

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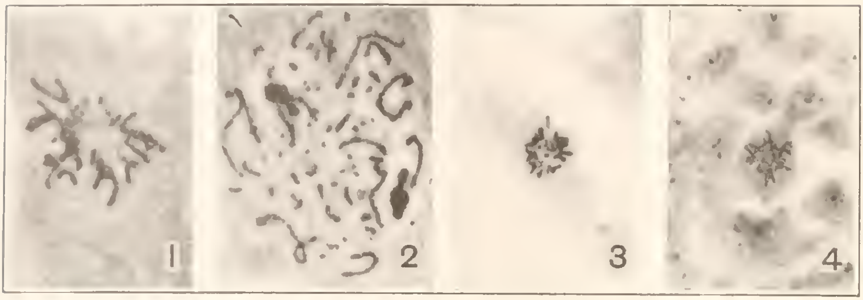
Those accustomed to working with tissue cultures are, I am sure, seldom troubled with doubts as to the essential normality of the processes going on in these isolated bits of tissue. Others, less familiar with the results and conditions of this type of work, usually make cautious reservations and distinctions in discussing phenomena occurring in living substance under its usual associations and when subjected to the technique of the tissue culture. Through the interest and courtesy of Mrs. M. R. Lewis of the Dept. of Embryology, Carnegie Inst. of Washington it has been possible for me to study mitosis in a number of beautifully fixed preparations of cultures of chick tissues. As this led later to an examination of this process in chick embryos a comparison was inevitable and a note concerning the characteristics of dividing cells as found in tissues existing under the two environments seemed desirable.

As regards fixation the tissue culture is infinitely easier to kill satisfactorily than a larger mass of tissue. The cells in the culture grow out over the cover glass usually in a single layer and the fixing reagent can come in contact with each cell individually and at once. This is, of course, an ideal which can never be approximated in material that must be sectioned. Consequently many fixatives which give excellent results with cultures are worthless when used on bulkier masses of cells. The size of the cells in the culture is another item in their favor for with the release from the crowded quarters of their usual surroundings they spread over the cover glass and press very closely to it, causing the diameter of the cell to increase greatly, associated with a marked thinning in the plane perpendicular to the cover glass. The cell becomes consequently much easier to see through and its various parts appears spread out in diagrammatic fashion.

There is also an increase in the size of the chromosomes as can be seen by comparing Fig. 1 with Figs. 3 and 4. This is an actual increase in size and may be caused by the increased or richer nourishment that it is possible to offer cells reared in cultures rather than by the mere flattening of the cell and its contents. It seems apparent, although but few accurate measurements have been made, that whatever has caused the chromosomes to increase in size has affected all equally. This observation is in harmony with the results reported elsewhere (Hance, 17 and 18) where it was demonstrated that an increase in the total amount of chromatin in a given cell was the result of a proportional increase in the size of each individual chromosome.



Photographs of dividing chick cells. × 1000.

FIG. 1. Late prophase of mitosis of cell growing in tissue culture.

FIG. 2. Prophase of mitosis of cell in tissue culture. Note the extreme flatness of the arrangement of the chromatic particles.

FIGS. 3 AND 4. Polar views of metaphase plates found in sectioned chick embryos. Compare their dimensions with Fig. 1.

The prophase stages of division which under ordinary conditions are like a ball of threads are in the cultures spread out so flatly that every granule of chromatin in the nucleus can frequently be recorded by photomicrograph as readily as can be the chromosomes of a very flat metaphase plate (Fig. 2). As the cells in the cultures are so exceedingly thin division never takes place perpendicular to the cover-glass but by the time mitosis has reached metaphase the chromosomes rearrange themselves so that division occurs in the plane of the cover. Consequently polar views of the metaphase cannot be found in cells in cultures although the very late prophase is so similar that for purposes of enumeration of the chromosomes it is quite satisfactory. Centrosomes and spindles are quite similar in cells dividing under the two conditions although they are clearer and possibly larger in the cultures.

An examination of the chromosome number has developed no distinctions between the two types of material. In the prophase of the culture cells the number of chromatic individuals is very large (from 60 to 70 in some cases). Prophases in which all chromatin rods or bodies can be counted are somewhat rare in sectioned embryonic cells but enough have been observed to indicate their entire similarity to the former. As the metaphase stage is approached the number of distinct chromosomes becomes less, presumably through the coalescence of certain chromatin granules or rods which previously had seemed to be separate. (See Fig. 2 for various series of chromating granules lying in straight lines which probably coalesce later in the mitotic process to form single chromosomes.) This results in a metaphase chromosome number that probably is between 35 and 40, it being difficult to determine the exact number owing to the minuteness of the smaller chromosomes. Nothing unusual develops in the culture anaphases and division is completed as normally there as in the embryo.

The tissue culture divisions were originally called to my attention by Mrs. Lewis because the chromosomes seemed to show some evidence of fragmentation such as I had reported finding in other forms (Hance, '17 and '18). Certain cells did possess a larger number of chromosomes than others. As already stated above the larger number is apparently an early prophase condition and the number decreases as the cell approaches metaphase through the union of what, in the prophase, have seemed to be discrete bodies. This condition occurs in embryos as well as in cultures. There is a little evidence drawn from instances where occasional somatic chromosomes are apparently without mates identical in form and size that suggests the possibility of fragmentation. However, considering the quite definitely determined condition discussed above of the running together of prophase granules to form chromosomes the asymmetry of the mates may as well, or perhaps even more likely, be due to the non-completion or total failure of the coalescing process as to fragmentation.

The retention of chromosome identity or individuality in the culture raised cells is also obvious. Fig. 5 shows a series arranged according to length, of the longer chromosomes of the mitotic complexes from cells found in a sectioned embryo and in a tissue culture. The smaller chromosomes of each cell have been omitted for convenience in printing and because, being short rods, they show no easily distinguishable features. A slight variation between the two sets of chromosomes illustrated enters through it being impossible, as pointed out above, to find a polar view of a metaphase plate in tissue culture cells. The lower series of chromosomes is taken from a very late prophase (tissue culture), so late that many of the chromosomes have assumed characteristic form. Number (from left to right) 3 has not yet taken on its final form. With this exception in mind it can be seen that the form of corresponding chromosomes in the two series and that the comparative size relations of a given chromosome to others in the same series or in the other series are identical. The possible causes for the chromosomes of the culture cells being larger have been discussed above.

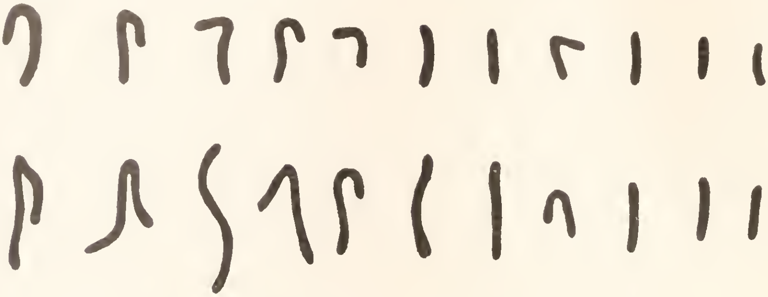


FIG. 5. Camera lucida drawings of the longer chromosomes of the chick complex arranged according to length.

Top row—from sectioned embryo.

Lower row—from tissue culture cell.

It is possible then to conclude that tissue cultures, as far as the mitotic phenomena are concerned, are quite normal and differ from these processes in the body only in minor reactions to the physical limitations of their surroundings.

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THE IRON-ACETOCARMINE METHOD OF FIXING AND STAINING CHROMOSOMES.

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A short description of this procedure was given in Vol. 55 of the *American Naturalist*, pp. 573–574. The method has now been used almost daily for five years, and certain improvements have suggested themselves.

The solution can be prepared by mixing about equal volumes of glacial acetic acid and water. To this is added powdered carmine in excess. The liquid is then just brought to boil, cooled, and decanted. To the red solution there are then added a few drops of a solution of ferric hydrate in 50 per cent. acetic acid, to act as a mordant. The amount of iron varies with different objects. Too much produces a precipitate in a short time. The more iron, the darker and bluer is the stain. A bluish red is usually the best. Large cover-glasses (20 by 50 mm.) should be used, and they should not be thicker than 0.17 mm. (Small or thick covers prevent pressure being properly applied to individual cells.) There should be a minimum thickness of liquid under the cover-glass. The edges can be sealed with a honey-thick solution of dammar in xylol applied with a brush; or better perhaps, with melted soft paraffin.

The following varied modes of preparation are useful in different cases.

1. Pollen-mother-cells, etc., are obtained free by pressing or tapping cut segments of anthers in a drop of iron-acetocarmine.

2. The cut-up anthers, or minute fragments of animal ovaries or testes, are put in a tube with a *large* excess of iron-acetocarmine. After 2 to 7 days, preparations are made in the usual way. This gives good results with cancer tumors.

3. Smears of large anthers or testes are made on a cover-glass, which is then laid on a drop of iron-acetocarmine on a slide.

4. For staining the vegetative and generative nuclei in pollen-grains or pollen-tubes, etc., crystals of chloral hydrate are dissolved in a few drops of iron-acetocarmine until the liquid just

clears the specimens sufficiently. (Too much chloral hydrate will cause shrinkage.) The pollen-grains are squeezed or partly crushed in this liquid, and may be left to stain for a day or two.

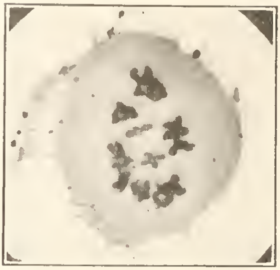

FIG. 1. Hyacinth—$2n$.

In the case of pollen-mother-cells, local pressure, with a small roll of paper, on the cover-glass, often spreads out the cytoplasm on the cover-glass or slide, with the chromosomes uninjured. If required, these can be preserved in balsam, by replacing the liquid by graduated mixtures of 45 per cent. acetic and alcohol. Fig. 1 is a balsam preparation of the reduction metaphase in a hyacinth, prepared in this way.

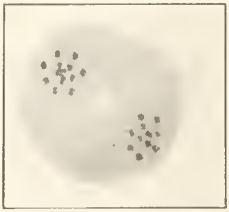

FIG. 2. *Datura*—$2n$—1.

The cytoplasm becomes quite clear in the acetic acid, and with an appropriate green screen on the microscope, the bluish red chromosomes stand out jet black. (Wratten green films Nos. 66 and 56, are most useful on a binocular, and No. 58 on a monocular, with a good tungsten electric light.) Fig. 2, a pollen-mother-cell of *Datura* in the second metaphase, with 23 chromosomes, in iron-acetocarmine, shows the sharpness of the images.

Pressure is usually best applied from 2 to 7 days after mounting, but the time varies for different pollen-mother-cells. Well prepared slides will often keep for some months.

The following notes may be of use.

Canna.—The pollen-mother cells are large and tender. The anthers should be cut into segments about 1 mm. long, and gently pressed out in a large drop of iron acetocarmine, being left ten minutes or so to toughen before putting on the cover-glass. (Here, of course, as usual, the anther remains are previously removed.)

Zea.—In maize the pollen-mother-cells are delicate, and the same process is required as for *Canna.*

Nicotiana.—In tobacco the pollen-mother-cells are easily pressed out and stained.

Datura.—Resembles a small tobacco. Pollen hard to stain.

Hyacinthus.—Yields excellently stained pollen-mother-cells, and also pollen-grains. The latter require several days to stain well. Pressing out is difficult.

Triticum and Secale.—In wheat and rye all the pollen-mother-cells are connected and come out of the anther loculus in a string. The young anthers are cut across once, and pressed out under the cover-glass. The pollen-grains stain well.

Tradescantia.—Stains deeply and shows chromomeres well in pollen-mother-cells, and in pollen-grains.

Uvularia.—Shows chromosomes like those of the Orthoptera in the pollen-mother-cells. Pollen stains well. Some metaphase chromosomes of the reduction division show compound rings.

Hosta.—Shows large and small chromosomes, the former with marked chromomeres. Stains well.

Cypripedium acaule, and C. pubescens.—Both pollen-mother-cells and pollen-grains stain well. Ten pairs of chromosomes.

Scilla sibirica.—Pollen-mother-cells stain well. Shows six pairs of chromosomes.

Narcissus.—Pollen-mother-cells and pollen-grains stain well.

Galanthus.—Pollen-mother-cells stain remarkably easily and well. Several different sizes of chromosomes.

The above plants have chromosomes which are especially worth studying, because of their size and for other reasons. In *Brassica, Gladiolus, Iris versicolor, Asparagus, Dahlia, Capsella, Hemerocallis, Antirrhinum, Stizolobium, Phaseolus* and *Crocus*, the chromosomes are either small, or clumped and not easily unravelled.

STUDY ON THE HABITS OF THE CRAB *DROMIA VULGARIS* M.E.

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The crab *Dromia vulgaris* usually carries on its back a sponge which it holds with the especially modified 4th and 5th pairs of ambulatory legs. The sponges, found on crabs at Villefranche, belonged to very different species. Only in the minority of cases did the sponge belong to the species *Suberites domuncula*, although the symbiosis of *Dromia* and *Suberites* has become nearly a classical instance. The sponges may belong to both the Calcarea and Incalcarea groups and very often *Dromia* covers its back with colonies of Ascidians, Algae, and even with pieces of paper or rags of any possible color. The fact of the non-specificity of this symbiosis was known to Polimanti and I believe that in reality there is no true *symbiosis* at all, as the sponge seems to be just as accidental a material as paper or rags. There is no doubt that all the activity is on the side of the crab.

The sponge ordinarily is astonishingly adapted to the size of the crab and to the shape of its back, especially in young individuals. It seems to be an exact copy of the back's surface. The cause of such a conformity is not exactly known. Renier uttered the hypothesis that the larva of *Suberites* becomes fixed on the back of a young *Dromia* and then both organisms grow together which accounts for the conformity of shape. However, the thing seems to be utterly improbable as there is no specificity in the "symbiosis." The pieces of paper may be just as well adapted to the size and to the shape of the crab as the sponge and thus even if the theory of Renier proved to be true, it cannot account for all the facts. Moreover the sponge is never fastened to the back, but it is simply held by the crab.

According to Vosmaer *Dromia* may tear off *Suberites* which are fastened to the convex shells of molluscs, obtaining in this way a concave surface that it puts then on its back. According to my own observations, this statement is fairly correct. I might add that *Dromia* can do the same with any kind of sponge, fastened to any immovable object. But in this case the hypothesis of Vosmaer becomes not quite adequate—as the sponge, torn off from a large stone, may be perfectly flat and there will be no conformity in shape. As far as the writer is aware the process of cutting and tearing off of the sponge by *Dromia* has not yet been described. The following observations and experiments pour some light on the whole question and they demonstrate at the same time the complication and the variability of the behavior of the crab.

The Process of Manufacturing a Case.

The process of manufacturing a case by *Dromia* may be easily observed if we put in the aquarium, which contains a crab, a comparatively large piece of ordinary writing paper. Before putting in the paper we remove the sponge from the crab's back. The paper must be boiled for a few minutes in water, otherwise it would float.

As a rule very soon *Dromia* grasps the edge of the paper with its claws and begins the work. The crab enters under the paper, holds the edge tightly and turns with its back to the ground. Now by means of rhythmical movements of both chelæ the crab cuts out of the paper a piece which is fairly similar in size and shape to the removed sponge. Nearly always *Dromia* starts its work from the edge. To state it more exactly, the crab does not cut the paper, but it tears it, and therefore the edges of the new case are always very uneven. Both chelæ grasp the paper until their points nearly touch one another, at the same time their planes are perpendicular to the edge of the future case. At the next moment both points are directed forward (upward if the crab lies on the back) and the paper between them tears. At once *Dromia* grasps the paper a little farther and tears it again in the same way. The animal pursues its work systematically and it keeps working always in a definite direction. As the border of the resulting case is approximatively elliptic, the mutual position of the chelæ cannot be always the same, but it varies according to a definite rule. It often happens that the crab pulls the paper too strongly and the edge becomes torn farther than necessary. But very rarely is the future case damaged in this way, nearly always such tears are directed outwardly. It is rather important to note that the animal does not pursue its work along every cleft but it chooses the right direction, correcting it every time. The clockwise direction of the work was just as frequent as the opposite one.

The legs of 2d–5th pairs participate indirectly in the work. They hold the paper and present it to the chelæ. If the crab does not stop in its business, which occurs from time to time, the process of cutting a case lasts about 15–30 minutes on the average.

The mode of working described is very frequently to be observed; however, it is not the only one possible. Sometimes before the cutting of the case is finished the crab grasps it with the hooks of the 4th–5th legs and puts it on the back. The case remains bound to the main mass of the paper. But after some time the work is always completed. Still holding the paper on the back *Dromia* may grasp the bridge, which joins the case with the rest of the paper, and tear it. The easier way is to release the case, to turn with the back towards the ground and to finish the work in the same way in which it was begun. It sometimes happens that the crab puts the whole paper on the back without cutting the case. Then the edge of the paper lies at the level of the forehead of the animal and the eyes remain uncovered. Rarely *Dromia* enters under the paper, grasping it near the middle. In both cases after some time the animal always starts the work of cutting a case of suitable size. Ordinarily *Dromia* releases the paper, turns on its back and begins to work as described above. If the first tearing off of the paper occurs in the middle of the whole piece, not at the edge, then *Dromia* folds it twice with the chelæ and tears it on the so-formed new edge. If the paper bears a small hole the crab uses it as the starting point of the work. Less frequent are the cases when *Dromia* keeps its normal position, holding the paper on its back, and without turning begins the cutting. The chelæ tear off one little piece of paper after the other, while the animal slowly revolves under the paper, grasping it every time at a new spot. During all this the crab retains a firm grasp on the paper. Nearly always *Dromia* works at the middle of one edge of the square, much more rarely the animal cuts off one of its angles. But it is only extremely seldom that the new paper case lies unsymmetrically. It seems probable that the equilibrium plays an important rôle therein.

During two months each of my crabs has been observed several times while working at its new case. There is some evidence that every individual has its own system of working. Four typical modes of cutting may be distinguished. The case is manufactured:

A. In the middle of the longer side of the rectangle.
B. In the middle of the shorter side of the rectangle.
C. At the corner.
D. Somewhere unsymmetrically.

Those four positions are to be seen in Fig. 1. In Table I. I have put together how many times every individual cut the case in each of the above four positions. Only few crabs, as Nos. VII. and IX., did not show any regularity of working. The remaining 8 individuals showed a marked predominance of one mode, especially if we take into consideration that the modes *A* and *B* are very similar.

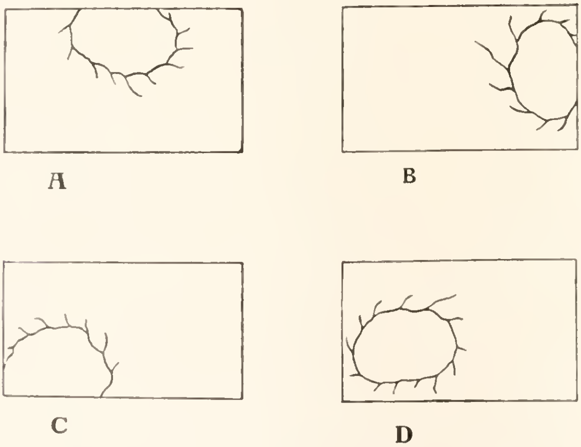

Fig. 1. The four modes of cutting a paper-case.

TABLE I.

	I	II	III	IV	V	VI	VII	VIII	IX	X	Total
A.........	0	10	11	0	9	1	3	0	8	1	43
B.........	3	4	8	2	9	1	6	20	7	16	76
C.........	16	3	0	18	0	14	5	0	3	2	61
D.........	1	3	1	0	2	4	6	0	2	1	20

The letters refer to the four modes of cutting. The Roman numbers designate individuals, the Arabic ones—the number of times for every crab and every category.

Never did the maximum number belong to the category *D*, which means that in 180 cases out of 200 *Dromia* started the work at the middle of one side of the rectangle, or at the corner. Some individuals, as IV., VI., VIII. and X. showed a very striking predominance of one definite mode of cutting and to a certain extent it may be concluded that a definite fashion of working is connected with definite individuals.

Similar modifications of the mode of working were observed when *Dromia* had to cut its case out of a sponge instead of the paper. The sponge was taken from a larger individual and given to a smaller one. The crab always cuts the sponge round and adjusts it to its own size and shape. The sponge is much thicker and less compact, as compared with the paper and therefore the crab was obliged to tear off small pieces of it instead of tearing it as a whole. The pieces, detached from the sponge were then directed towards the mouth where the maxillipedes held them for a while and then let them drop; the current which brings the water to the gill-chamber carried them away. As in the case of paper, *Dromia* may perform its work while lying on its back or in the normal position of the body. The sponge is held tightly by means of 4th–5th pairs of legs. Sometimes the crab will carry on its back a sponge which is much larger than the normal case and will start the work only after a considerable time. But sooner or later the sponge always becomes adjusted to the size of the animal.

A very interesting reaction occurs when we put into the aquarium a large stone covered with a sponge. The animal very soon climbs upon it, grasps the sponge at two points, and tries to tear it off from the stone. After some unsuccessful attempts it begins to work systematically, tearing off small pieces of sponge along a fairly regular elliptical line, while it turns slowly at the spot. A regular elliptical furrow results, which goes through the whole thickness of the sponge reaching down to the surface of the stone. This furrow is the outline of the future case. Now *Dromia* grasps the periphery of the furrow at two points and pulls it towards itself trying to tear the sponge off. This movement differs very distinctly from the behavior of *Dromia* while cutting the sponge through. As the sponge holds very tightly this attempt usually remains without success. Following this *Dromia* begins again to tear pieces of sponge, but now it works along the furrow trying to press the chelæ between the sponge and the stone. Sometimes the animal proceeds in one direction towards the center of the sponge, remaining at the same spot during the work, but often it detaches the edge of the sponge from the stone along the whole circumference of the furrow. In the former case it starts the work from below, sitting on the future case and directing its head downward. All the time the planes of both chelæ remain parallel to the surface of the stone and the work now is much more a systematical detaching than cutting, as only few pieces of the sponge are now torn off. After some time the crab tries again to tear off the whole case, grasping the edge of it at two points and pulling it towards itself. Usually this second attempt is made at the right time and the animal, holding the sponge tightly, rolls with it from the stone to the bottom of the aquarium. If however, the attempt was premature *Dromia* returns at once to the interrupted work and after some time repeats the same attempt once more. Sooner or later there always results a case corresponding very exactly to the size of the animal.

It seems that a suitable consistency of the sponge, rather than the curvature of the stone corresponding to the curvature of the animal's back, decides the choice of the spot where *Dromia* starts its work. Therefore very often the manufactured case is too flat and it does not fit exactly to the surface of the back. The crab corrects this by pressing the sponge tightly to the back and bending it out. As the tissues of the sponge are fairly plastic, the resulting curvature of the sponge becomes somewhat fixed after some time and it remains unaltered even if we remove the case from the animal's back. I noticed repeatedly that the coarse inner surface of the new case soon becomes smooth and resembles closely the inner surfaces of natural cases found in the sea. Very probably it depends once more on the mentioned plasticity of the sponge.

If the stone, which we put into the aquarium, was covered by several distinct sponges of different size, but of somewhat rounded circumference *Dromia* did not cut any of them, but chose the most suitable one and tore it off as described above.

It remains not fully explained why larger specimens of *Dromia* usually carry cases on them which cover only the posterior part of the body and are too small. Those sponges have very often uneven edges and generally they fit much worse than the cases of young individuals. I believe that the comparatively small cases result from the difficulty of finding sufficiently large sponges which may be torn off without being damaged. Younger crabs can use sponges which are smaller and therefore younger and more plastic. Perhaps it accounts for the fact that only the cases of small *Dromia* are usually admirably fitted.

Putting of the Sponge on the Back.

After having manufactured a case *Dromia* puts it on the back. This process proved to be an extremely various one, which depends largely on the random position of the sponge.

1. If we take the sponge off from the crab's back and put it in the aquarium with its concavity directed upward, the animal usually returns to it very soon. *Dromia* grasps the sponge from both sides with the fore legs (Fig. 2*A*) and falls on its back, pulling the sponge over it. The sponge, supported by the legs, lies now with its concavity directed downward (Fig. 2*B*). Lying still in this position the animal begins to turn and to move its case in a horizontal plane until the fore edge of it, in the normal position of the animal directed towards the head, points to the abdomen. An ordinary case has an elliptical circumference and its longer axis lies at right angles to the sagittal axis of the body; the hind edge is marked by its greater curvature, while the fore edge is somewhat flattened.

In the next stage of the process the crab grasps the sponge with the hooks of the 4th and 5th pairs of legs and changing the point on which the hooks hold the inner surface of the case as the crab moves it, lifts its abdominal end above the bottom (Fig. 2*C*) until the longitudinal axis of the body lies vertically and the

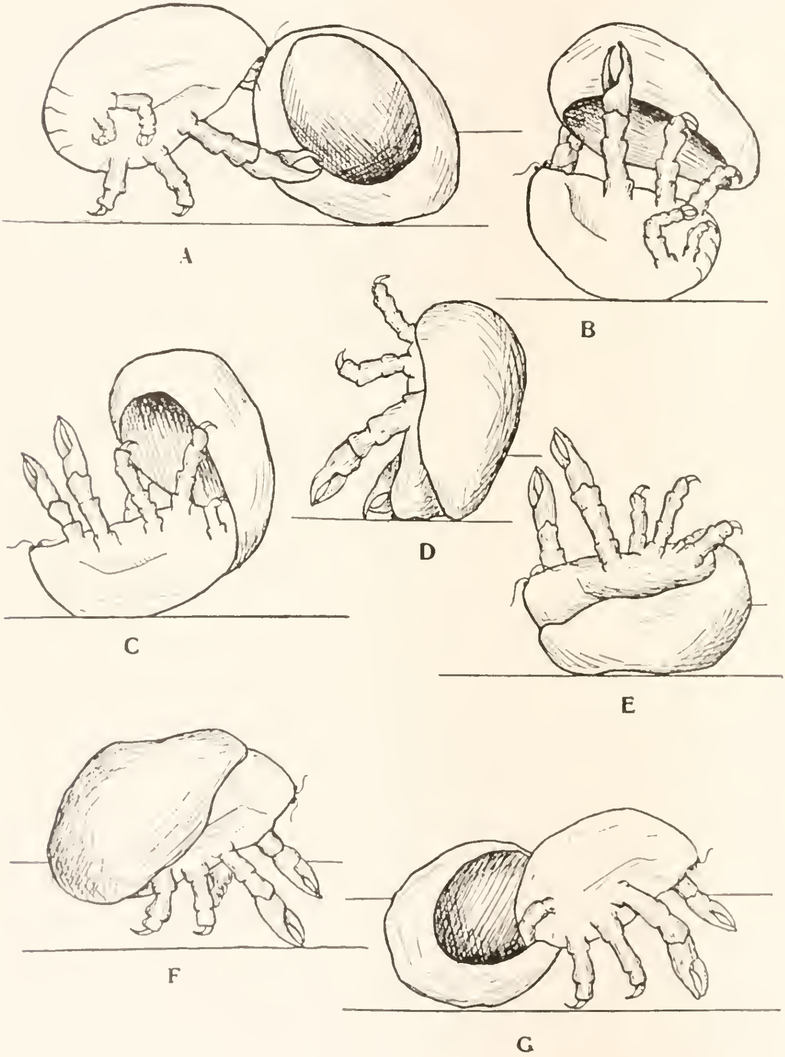

FIG. 2. Different modes of putting the sponge on the back. For explanation see the text.

animal stands on its head (Fig. 2*D*). At the same time the position of the sponge is gradually altered: its fore edge becomes inclined towards the abdomen and the sponge becomes almost vertical in position touching the ground with the fore edge (Fig. 2*D*). As a consequence the abdominal end of the body moves along the concavity of the sponge upwardly and, as the back of the animal remains pressed tightly to the sponge, *Dromia* while standing on its head assumes its normal position towards the sponge. This means that the fore edge of the sponge touches the head. The vertical position of both crab and sponge is of course, a position of an unstable equilibrium, and therefore either the animal falls forward, assuming the normal position, or it falls on its back. In the latter case it can turn in different ways, as described in the paper of Dembowski.

The grade of lifting of the abdomen above the ground may be very different and sometimes the position of the longitudinal axis of the body may be only slightly oblique towards the bottom of the aquarium.

2. In other cases, *Dromia* approaches the sponge, turning its abdominal end to it. The sponge still lies with its cavity directed upwardly. When the abdomen touches the edge the hooks of the 4th and 5th pairs of legs grasp the inner surface of the sponge near its edge (Fig. 2*G*) and then suddenly contracting these legs, the crab simply flings the sponge on the back. In the majority of cases the animal approaches the sponge at the right spot, in others it turns the case correspondingly, either before putting it on the back, or later, in its definite position.

3. There occurs sometimes a modification of case 2. The crab approaches the sponge directing its head towards it, climbs over the sponge and goes farther still in the same direction until the fore end of the body touches the ground on the opposite side of the meeting point (Fig. 2*G*). When only the abdominal end remains in the concavity of the sponge the legs of 4th–5th pairs grasp its inner surface near the edge and fling the sponge on the back.

4. In still other cases *Dromia* enters the sponge (always lying with its concavity directed upwardly) and turns on the back, as shown in Fig. 2*E*. The animal changes its position towards the

sponge several times and at last it always succeeds in assuming the normal one in which the head touches the fore edge of the case. The hooks of the 4th–5th pairs of legs, as usual, grasp the inner surface of the sponge and the animal turns forward to its ordinary position, pulling the sponge behind itself.

In all described cases *Dromia* puts its own sponge on the back in a correct position. But if the sponge belonged to another individual, the position of it becomes often irregular, it may even be put on with its concavity directed upwardly. Still after some time the position will always be corrected.

5. The behavior of the animal changes a little if the sponge is lying with its concavity downward. In the majority of cases it will be grasped with both chelæ and, as before, the animal, falling on its back, pulls the sponge over it. Now the crab turns the sponge not only in a horizontal plane, but holding it with 1st–2d pairs of legs it turns its concavity downwardly. After this the behavior becomes similar to the case 1.

6. *Dromia* may press the abdominal end of the body between the sponge and the ground (the concavity of the sponge being turned downward), then it enters under the sponge and grasps its inner surface with the hooks of the 4th–5th legs. The sponge becomes adjusted at once, or it may be turned correspondingly in a horizontal plane.

7. Before attempting to put the sponge on its back the animal may turn its concavity upward and then it behaves as usual. This turning occurs while *Dromia* pushes the edge of the case with the forehead, or it presses the chelæ between the edge of the sponge and the bottom. If there are some small stones on the ground the task becomes easier, as the stones prevent the gliding of the sponge along the bottom, furnishing at the same time a certain point of support for the animal.

Dromia is able to surmount many unexpected difficulties when putting the case on. If we put a few stones in the sponge, which lies with its concavity directed upward, the sponge becomes too heavy and the animal cannot turn it. After some time however the sponge always will be liberated from the stones. In most cases *Dromia* attempts at first one of the described above modes of acting, as if there were no difficulty at all. But as those attempts will be unsuccessful now, the animal presses its fore or hind end between the sponge and the ground until it overthrows the case and causes the stones to fall out. We have seen already that the crab behaves sometimes in that way when the sponge lies with its concavity downward. It will be noted, however, that the animal never does this when the sponge points with its concavity upward and there is no doubt that this behavior is caused by the impossibility of using one of the ordinary ways.

In other cases the stones will be pushed with one chela towards the edge of the sponge until they fall out. This work is but seldom carried to an end and some of the stones usually remain when the animal puts the sponge on its back. The remaining stones prevent the proper adjustment of the sponge and after some time *Dromia* repeatedly lifts the sponge over its back and presses it again, which finally causes the stones to fall out, slipping between the back of the animal and the inner surface of the sponge.

Dromia can manage the liberating even in cases when the sponge remains completely covered by the stones and invisible. This requires however some previous learning. Every time that the crab succeeds in putting the sponge on the back, we take it off and put still more and more stones into it, keeping the sponge always at the same point of the bottom. After some time *Dromia* becomes accustomed to the place where the sponge lies and it goes directly towards it, trying to move the stones and liberate the sponge. This behavior becomes striking when, after several repetitions, we put the sponge in quite a different place. The animal goes nevertheless directly to the previous spot and begins to seek the sponge. As control experiments showed, it never behaves in this way without previous learning.

In another series of experiments I fastened the sponge to a little wire hook. In the meantime the crab manufactured a new case of paper. In order to make the sponge more conspicuous I chose a red one and put it into a porcelain dish, hanging it so high on the wall that the crab could hardly reach it. When I put the crab in, it directed itself at once towards the sponge, *dropping the paper case on the way.* With some difficulty the animal succeeded in grasping the hanging sponge with the chelæ.

It then climbed over the sponge, very soon found the wire hook, and began to tear off small pieces of sponge around it. After a short time the sponge became liberated and the *Dromia* rolled down with it, and put the sponge on the back. From the moment of putting the crab into the vessel to the liberation of the sponge 7 minutes elapsed. I repeated this experiment several times and I found that the described behavior is a typical one. Shortly after liberating the sponge I took it off again, giving to the animal the paper case which was accepted. Then the sponge was again attached very tightly to a wire hook and was hung on the wall. After being put into the vessel, the crab once more directed itself immediately towards the sponge, climbed over it and only now dropped the paper case. The next moment it adjusted itself in the sponge, grasping its inner surface with the 4th–5th pairs of legs and pressing the back to the concavity of the case, assuming its normal position. Now the animal tried to go with the sponge, but naturally succeeded only in gliding with the legs on the smooth wall of the vessel. Then it changed several times its position in the sponge, still holding it tightly, but only after two hours of continuous attempts it found the wire hook, with the chelæ. Now the liberation took only few minutes and as before *Dromia* rolled with the sponge to the bottom. I repeated this experiment with the same individual over 60 times in about 20 days and found that in the great majority of cases the animal directs itself immediately to the wire hook and only very rarely, and during a short time, it tries to go with the sponge on the back. The time of liberating varied from 6 to 20 minutes, on the average it was 8 minutes. Many other individuals showed exactly the same reactions. After some time, ordinarily after a few minutes, each crab succeeded in finding the wire hook and liberating the sponge.

It is to be noticed that *Dromia* possesses a marked faculty of choosing the material for its case, which depends somewhat on the past of the animal. Several facts corroborate this conclusion.

1. We take off the sponge from a fresh *Dromia* and we put into the aquarium several different materials, such as a sponge, an artificial case made of plastelin, a piece of rag, paper, etc. Under those conditions, the animal chooses the sponge. In most cases it recognizes its own case from many other sponges.

2. If the crab has carried an artificial plastelin case on its back for a long time, it often happens that it chooses its own plastelin case, even if near to it there is a sponge of suitable size.

3. *Dromia* may be found without any case at all. Such an animal always chooses a sponge, and so does an individual which remained in the aquarium uncovered for a certain time.

4. We give cases of different kinds to an individual, which carries its own case on the back. If the latter was a paper case, then *Dromia* always drops it and chooses a sponge. Under the same conditions a case of plasteline was dropped only sometimes, but a normal case of sponge was nearly always kept by the animal.

Conclusions.

To a certain extent the described facts furnish an answer to the questions raised at the beginning of this paper. It follows certainly from them that there is no reason to speak about symbiosis of *Dromia* and sponge, as the mutual adaptation is entirely lacking. If the crab is especially adapted to carry a case on its back, the sponge surely remains completely passive. The lack of any specificity of the symbiosis proves it very clearly. It is not plain what may be the use of the sponge to *Dromia*. Polimanti put covered and uncovered *Dromia* into an aquarium containing an *Octopus*. He found that the uncovered individuals were devoured, just as any crabs of some other species, but the covered ones remained safe. However Polimanti does not decide whether the *Octopus* simply was unable to see the covered animals, or whether the sponge held it away. At all events, as far as the optical side of the problem is concerned, it may be interpreted in different ways. In most cases there is no correspondence in color between the sponge and the surroundings. The sponge, which *Dromia* carries on it, may be of very different, often bright, colors: red, yellow, purple, etc. The presence of the sponge even facilitates the catching of *Dromia*, as the crab usually remains on vertical walls very near to the surface of the sea. An uncovered specimen is less conspicuous by far, on account of its uniform gray or brownish coloration. But just as frequently the sponge may be of a somewhat protective coloration, gray, brownish, or greenish, which contradicts again the theory of a warning color. It will be safer probably to conclude that the color of the sponge is an accidental one and that it does not serve any special purpose.

The conformity of the inner surface of the sponge and of the back of *Dromia*, especially in young individuals, may be explained without any artificial assumptions, like the theories of Renier and Vosmaer. We did see that the animal possesses the faculty of cutting a sponge of suitable size and circumference, tearing it off from a stone, and putting it on its back. As the sponge remains then pressed tightly to the back of the crab, it soon assumes a suitable shape. When moulting the crab must drop the sponge. But we know that all the crabs after the moult remain nearly motionless until the new chitin hardens. We know moreover, that *Dromia* can find a sponge which is entirely covered by stones, and that it can dig it out. It follows therefrom, that very often at least after the moult the crab may pick up its own sponge again. But still we do not know exactly if the rapidity of growth is the same in both organisms. At all events it must happen very often that under its natural life conditions *Dromia* is compelled to find a new case for itself.

The described facts confirm once more the very complex character of reactions of a crab. The behavior of the animal towards the case is a series of most complicated movements, too complicated in fact to be explained by simple external stimulations. From the physiological point of view there exists the possibility that the pressure of the case on the sensory hairs of the animal's back and the effect of its weight on the 4th–5th pairs of ambulatory legs form the stimulus which inhibits the movements leading to the finding and manufacturing a new case. Conversely, the lack of such stimulus may be one of the causes of those movements. But there exists an interesting fact that contradicts the above assumption. One individual, while carrying on its back its normal sponge case, began to manufacture a new case from a piece of paper, without dropping its sponge. Such facts are rare, it is true. However, it happens more frequently that individuals, which are carrying a case of plasteline or paper, choose a sponge and grasp it with the chelæ, and only now drop their cases from the back. This behavior may be called "senseless," but it proves that the stimulus, which compels the animal to act in a definite way is a complex one and that the optical side of the question plays an important rôle therein. We did see, in fact, that the crab rushes at once towards a distant sponge, if it is made conspicuous by putting it into a porcelain dish.

As in all the reactions of a crab, its behavior towards the sponge varies from individual to individual. In some cases, but not always, it may depend on a different activity of different individuals. There are marked differences, depending on the age. Older specimens are by far less active, and this bears on the whole behavior. The young crabs begin their work of cutting the case much sooner, the work itself is done more quickly, and the cases are made more carefully. They attack very often other individuals and take their cases away, which occurs only rarely in larger crabs. Those observations agree with the statement of Watson. This author found, in fact, that young rats, 23 days of age, possess already the faculty of performing all the tasks that a rat is able to perform, in spite of their nervous system being not yet definitely developed. On account of their greater activity, the young rats often solve different problems more quickly than the adult individuals.

Some facts reported above confirm the conclusions of Yerkes, Yerkes and Huggins, Spaulding, v.d. Heyde, Mikhailoff, concerning the existence of associative memory in the crab. If a crab carried for a long time a case of sponge or plasteline, and if this case be taken off from it, the animal nearly always chooses from several cases its previous one. The crab may learn to come to a definite spot in the aquarium, where its sponge is lying under the stones, even if this sponge be put somewhere else.

The reported observations are somewhat fragmentary. The problem of the conformity of shape of both organisms is not entirely solved by them, as we do not know what may be the behavior of very young crabs that are putting on a sponge for the first time. The behavior after the moult also has not yet been observed. Nevertheless the described facts do elucidate some points of the question and, which is perhaps more important, they show many experimental possibilities of studying the behavior of this most interesting animal.

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BIOLOGICAL BULLETIN

NOTES ON THE BEHAVIOR OF THE FIDDLER CRAB.

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There exists already a small literature dealing with habits of fiddler crabs and the general biology of these forms is known fairly well. As far as I am aware none of the various reactions of the animals, however, has yet been studied thoroughly. A *careful* study of the movements of an animal undoubtedly differs from a general one not merely in bringing some new details, but as well in bringing quite new problems. I quote Schwartz and Safir: "Correlated with the regular recurring changes in the tide, Uca performs its tasks with unchanging regularity, its general behavior never deviating from its standard, never altering from its established method, being almost stereotyped" (18 p. 20). "It has few tasks to accomplish, and does them day in and day out in the same way" (ibid.). From a certain point of view a crab has but a few tasks to accomplish, as digging the burrow, feeding, fighting and performing its sexual activities. From a similar point of view the "general behavior" of man is also a monotony of reactions, like dressing, undressing, eating, walking, talking and sleeping. However we do not consider the man as being a stereotyped automate because we know a lot about our own life, and we know that every one of those reactions may be performed in infinitely different ways. And yet a careful observation of the activities of a fiddler crab leads to very similar conclusions. Every individual digs its burrow but the details of this process may be infinitely various and the behavior of the animal is as far as possible from a stereotype.

My time being very limited I succeeded in observing but few particulars. Nevertheless they seem to indicate that the existing literature on the fiddler crabs has not exhausted all possibilities.

THE BURROWING INSTINCT.

All my observations were done on *Uca pugilator*, the sand-fiddler. In the environs of Woods Hole this species lives on sand banks communicating with the ocean by means of a complicated system of ponds and channels. On account of this circumstance the rising tide is very quiet and the animals are never exposed to waves. In fact so slow is the movement of the water that at the low tide one may see clearly sand-pellets removed by the animal from its burrow during the previous low tide. This particular is rather important to note, as many habits of *Uca* are correlated even with slow tidal changes.

Watching the animals in the field furnishes only data which are already known and there is no need to relate them once more. But one particular concerning the shape of the natural burrows deserves some attention. I studied it, pouring a solution of white plaster into the burrow and digging the mould out after it became hard. According to Pearse (12, 13) the burrows of *Uca pugilator* rarely exceed 75 cm., which I found to be correct. The burrows are nearly always oblique to the surface of the ground and they show a general tendency to become somewhat horizontal at the inner end. But there are very many modifications and it would be rather difficult to say which is the type. Sometimes the beginning of the burrow is vertical and then it bends sharply, as in Fig. 1, *a*; sometimes the inner end of an oblique burrow suddenly becomes vertical as in Fig. 1, *c*. They show, however, some common features, as they are always somewhat bent, never perfectly straight. Every burrow ends with a marked swelling—the end-chamber—where the animal often remains. In some cases the end-chamber is so large that its inhabitant may turn in it in every direction, in others it allows only turning around the transversal axis of the body. I failed to notice any difference connected with the sex of the crab. Also I have never seen a branching passage, as described by Cowles for *Ocypoda arenaria* (5). I found such a passage in some burrows of *Uca pugnax* but the burrow of *Uca pugilator* is always a simple tube. It is difficult to say to what conditions may be due the marked individual differences in the shape of the burrows, as the external conditions on a horizontal bank of pure sand seem to be perfectly uniform for all individuals. Referring them to the different individuality of the crabs may be true, but probably it means nothing more than our complete ignorance of the causes.

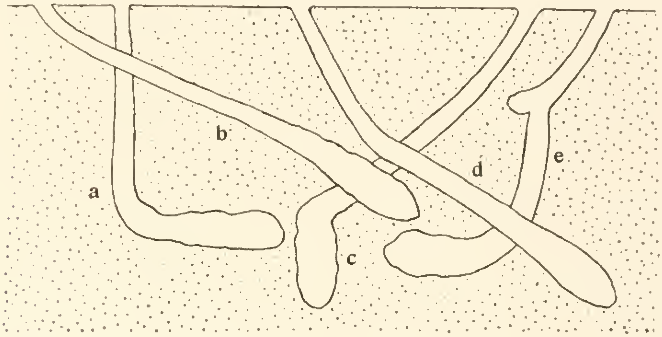

FIG. 1. Different types of natural burrows.

To be able to watch closely the process of burrowing we must observe it in the laboratory. In cylindric glass-jars (6 inches in diameter, 9 inches high) half filled with wet sand the crabs struggle madly and try to escape by climbing on the glass wall, in which they are of course unsuccessful. The animals remain near the wall and particularly near the most lighted spot of it. After some time the movements become slower, the crab gives up its attempts to escape and it keeps quiet for a while in its normal position. Finally it begins to dig. The individual behavior may be very different. Some crabs start their work after a few minutes, others roam around the jar for hours and even days. Some remain motionless for many hours, others move perpetually and struggle for escape. Some may walk around the wall showing no particular predilection for the lighted side, some again remain for a long time at the spot nearest to the window. There is a slight difference between the sexes, the females being more shy on the average and starting the work sooner. The mode of working is also somewhat different in both sexes, which will be mentioned later.

We come to our first question: on what conditions depends the spot at which the animal starts digging? The spot is not quite a random one as some rules seem to hold. Practically in all cases the hole lies close to the wall of the jar and at least very often the wall directed towards the light is preferred. The answer is a simple one. The crab is both positively phototactic and thigmotactic. During its struggling for escape *Uca* remains at the most lighted spot of the wall; there it quiets down and there it begins to dig. This explanation may be correct, but it leads to further considerations. Phototaxis means of course an overwhelming reaction towards the light, and only in this sense it has a definite meaning. We are also positively phototactic and we do not like to remain in the dark. Nevertheless nobody would explain the reactions of man on this basis as our phototaxis is checked by very many other reactions. Such an explanation, however true in some cases, would be one-sided and rather poor. But the same holds also for the crab. The animal is positively phototactic, and yet it digs a burrow which conducts it away from the light. During the burrowing *Uca* repeatedly comes out of the hole towards the light and it enters the burrow which is dark. Is then the phototaxis changing with every minute? In the jar the crab keeps usually close to the wall during the whole work and the whole burrow may be fully exposed to light, which does not disturb the animal in any way. After having finished the work and after having filled the burrow almost entirely with sand, as described below, the crab remains at the bottom of the jar, in the end-chamber, which is often fully lighted, and it may remain there motionless for days. We can compel the fiddler to dig in the middle of the jar making there a shallow hole and driving the crab into it. But as the burrow is oblique the animal soon reaches the wall of the jar at some particular spot and it makes an end-chamber there, being by no means disturbed by the light. We must consider that a similar situation never occurs under normal life conditions of *Uca* as the hole always leads to the dark. The natural burrows have their openings just as often directed to the south as to the north. All the evidences indicate very strongly that under fairly normal conditions of life *Uca* is insensible to light, or, to say it more correctly, phototaxis does not play any marked rôle in its life. According to Schwartz and Safir on cloudy days the fiddlers remain inactive. On the contrary, in my laboratory hundreds of fiddlers displayed the most activity in the dark which may depend on being undisturbed when nobody remained in the room. The inactivity on cloudy days is surely connected with heat, not with the light. What we call phototaxis in *Uca* may be observed only under very unnatural conditions when the crab is struggling blindly for escape. Its activities are then uncontrolled by the inhibitory centers and automatic correlations may drive the animal towards the light. Such cases are very interesting for a study of automatic nervous connections but they are scarcely valuable for psychology.

As to the thigmotaxis some observations point to its existence. In the dark *Uca* also dig their holes near the wall but of course no particular spot of it is preferred to the others. And again a control experiment shows that the thing is not so simple. I put several crabs into a large crystalliser (11 inches in diameter) filled with sand. I thrust in the sand several small glass plates at different spots, covered the crystalliser and put the whole in the dark. If remaining close to the wall be only a matter of thigmotaxis we might expect that the crabs will dig just as often near the glass plates scattered through the whole surface of the sand, as they dig near the outer wall. But in fact from 26 crabs tested several times only one made the burrow near a peripheral glass plate, all others dug invariably close to the outer wall. And yet the sum of contact surfaces of all plates with the sand was even superior to the surface of the crystallizer's wall. Once more the thigmotaxis proves to be not an adequate explanation. The tendency of the crab is not to remain by the wall but to remain at the very periphery of the vessel. Under similar conditions practically all animals show the same behavior, *Paramœcium* as well as man. As I showed (6) *Paramœcium* swims along the periphery of the vessel in spite of its thigmotaxis being negative while swimming. In a closed place which we wish to leave we will be oftener found at the periphery than in the middle of it. The reaction has nothing to do with thigmotaxis.

Besides photo- and thigmotaxis there is a strong hydrotaxis, the crab striving towards the moisture. There exists also a very marked tendency of hiding itself in the sand. Especially in females we may see often a behavior distinctly different from the ordinary burrowing. It consists in a quick pressing of the body in the sand. As the friction is very strong the animal succeeds only in hiding the thorax while the legs of one side of the body remain uncovered. In this position the crab may remain motionless for hours and such a behavior certainly has some biological significance as very little is seen of the crab, the legs having a marked protective coloration. The consistency of the ground has also some influence on the choice of the spot where the work is started. If we make a shallow hole in the sand *Uca* will choose it as the starting point. All those "taxies" and strivings, and many more, determine the actual behavior and referring the activities of a crab to some particular tropism is rather a poor explanation.

However, at last the animal starts its work. To simplify the nomenclature I shall use roman numbers I.–V. for the legs of the side of the large claw of the male, and the arabic 1–5 for the legs of the opposite side. Thus I. means the large chela, 1 — the small one.

At the very beginning of the work the side 1–5 is usually directed towards the wall of the jar, straightly or obliquely, and usually this side begins to dig. The legs 2, 3 and 4 which are bent in the articulations mero-carpopodit and dactylo-propodit at the normal position of the body become somewhat stiff. At the next moment they become bent a little more in both articu-

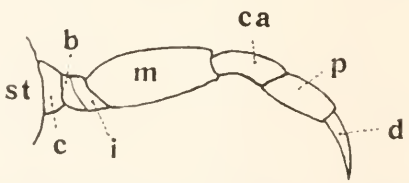

FIG. 2. The second leg from the side. *st*, sternum; *c*, coxopodit; *b*, basipodit; *i*, ischiopodit; *m*, meropodit; *ca*, carpopodit; *p*, propodit; *d*, dactylopodit. There is no movable articulation between *b* and *i*. Articulations *p–d*, *m–ca* and *c–b* work in the same plane, others at right angles to them.

lations mentioned above and the terminal hooks (dactylopodits) sink into the sand. The legs are not bent at once but one after another in a quick rhythm which helps to make the sand lighter. If the sand is hard those efforts are repeated, until the whole dactylopodits and a good part of propodits enter into it. A portion of sand lies now between the legs 2, 3, 4 and the side of the thorax. Now the legs become also bent in the articulation coxo-basipodit which works in the same direction as the former two. The portion of sand becomes loosened from the ground and tightly pressed to the side of the carapace. The crab walks a few centimeters aside carrying the sand, then it stops and the legs 2, 3, 4 become bent also in the articulation sternum-coxopodit working nearly at right angles to the former three. Through this movement the pellet is directed towards the sagital plane of the body and forward. It is pushed in this direction by the side surface of the working legs. A further bending of the same articulation causes the legs to touch the pellet with their external surface. As soon as this occurred the legs become extended in the articulation mero-carpopodit one after another as at the beginning. The pellet may be now pushed still further towards the side I.–V. The sand is not very wet at the surface and the pellet does not hold together. The lose sand grains are to be prevented from falling back into the hollow remaining at the place of the removed pellet and the crab does it very carefully. In this work of pushing away the sand which is not any more held by the legs the small chela helps also. Very often it starts helping even before, when the pellet is first formed, pushing it together with legs 2, 3, 4. But it is never used for digging in the proper sense. The leg 5 also remains inactive in digging as it has a different task to accomplish. After having pushed the sand away, removed the grains that remained on the legs and kneaded the sand a little the animal returns to the hollow and grasps another portion of sand in the same way. But even the second pellet brings some difficulty with it as it is never carried far away and the lose grains of sand easily roll back. Therefore as the work progresses, more and more legs participate in it. Besides the legs 1, 2, 3, 4 the leg II. begins to help. When the pellet is already pushed beyond the sagittal plane of the body the leg II. strongly bent in the articulation mero-carpopodit comes behind the pellet touching it with the external surface of pro- and dactylopodit. Extending the leg II. in the mentioned articulation the crab pushes and kneads the sand in the same way as do the legs 1–4. A little later also the legs III. and IV. are used in the same manner. Then the large chela begins to play its part. Its lower edge is held closely to the surface of the ground and extending the leg in the articulation mero-carpopodit *Uca* pushes from time to time large portions of sand away as with a spade. Many authors (Alcock, Pearse) consider the large claw of *Uca* as being a secondary sexual character. But it plays also an important rôle in digging.

The deeper the burrow becomes the higher is the sand hill at its mouth and the greater is the danger that the sand will roll back. All legs, excepting V. and 5, participate in pushing it away and this work is done more and more carefully. At the same time the sand from the deeper layers is more wet and the grains hold together better which facilitates the task. The pellets may be simply rolled away from the mouth of the burrow. In this action again all legs participate and the digging side presents the pellet to the pushing side which rolls it farther. The legs II., III., IV. push the pellet with the external side of their pro- and dactylopodits, the crab walking after it sometimes at a considerable distance. In the field the removed pellets often lie as far as one meter from the mouth of the hole. Sometimes when the chela I. has pushed a pellet far away it will do the same with some other pellets before the animal returns in the hole, as if the crab wanted to mend its former inaccurate work. The more sand is carried out the farther the crab rolls the pellet; sometimes it is deposited at the opposite side of the jar. The large chela, as mentioned, is used only from time to time; it does a rough work. In most cases it would only disturb the rolling of the pellets. Therefore it is always raised high in the air in the moment when the side 1–4 presents the pellet to the side II.–IV.

There is still the problem of locomotion to be solved. As known, during the sidewalk of crabs the legs directed forward pull the body while the opposite legs push it. As the digging work progresses the crab has to carry its burden from a greater and greater depth. The legs II.–V. are free in pulling the body, but the legs 2–4 are holding the sand. The chief rôle in the locomotion on the inner side is played by the leg 5 which is never used for burrowing. In the later stages of burrowing the leg 2 also begins to help a little. Its articulations pro-dactylopodit and mero-carpopodit are moving and pushing the body, while the meropodit holds the pellet. To a certain extent also the small chela helps. Only the legs 3 and 4 remain motionless, holding the sand.

Another interesting particular. When the burrow is just deep enough to hide the thorax but the legs I.–V. remain outside, the crab often grasps the edge of the hole with legs II.–V. helping to move the body out of the burrow. There is the danger that the edge will break under this effort and the sand will fall into the burrow. Thus the crab grasps the edge at four points possibly distant from one another embracing about 160° of its circumference. In all other cases the legs are held much closer together.

The burrow is perfectly circular in every transverse section. This becomes possible because the crab changes very often its position within the tube turning around the transversal axis of the body. As the canal of the burrow is oblique the animal sometimes walks along it having its legs directed towards the upper wall and the back touching the lower one. Consequently *Uca* comes out from the burrow touching different points of the hole and this causes the pellets to be deposited at different spots around it. But the most of them are deposited in the direction which is a prolongation of the inclined burrow. Evidently walking along the lower wall of the canal is easier and occurs oftener.

Still repeating all those described movements the animal succeeds in digging a deep burrow ending blindly at the bottom of the jar. However sometimes *Uca* stops digging before the bottom is reached. As mentioned above the inner end of the burrow forms a kind of chamber markedly larger in diameter as compared with the canal. The animal is especially careful in making it and often it will spend hours in finishing the end-chamber. The principal instrument is now the small claw which is repeatedly pressed with its external surface against the wall flattening the sand and hardening it. During the work the crab changes its position and it is often to be seen lying on its back and working at the roof. When the burrow is finished the animal will remain motionless, sitting in the end-chamber for a long time.

The female works in a closely similar way. But as both chelæ are equal there is no preference to either side of the body. In pushing the sand the claw of the side external to the burrow works as the other legs. From time to time the female will change the working side. On the average the work is accomplished a little sooner than in the male which is possibly due to the changing of the side digging which gives to it a chance for resting. One observation points in fact that the legs may soon become tired. When a crab runs along the sand for some paces it will stop from time to time quickly changing the side directed forward.

If the crab meets any obstacle while digging, its behavior depends very much on the stage of the work at which this occurred. Small stones are simply carried out like a sand pellet. Bigger stones which are too heavy for the animal cause an abandoning of the hole if they lay near the surface. In such cases the animal will start digging another burrow at some spot near to the first, changing the spot many times until it finds a suitable one. But if the obstacle lies deeper the efforts of the crab may last for a long time and often it rather prefers to change the direction of the burrow than to abandon a work which is half done. This has been observed already by Schwartz and Safir. The peculiar form of the burrow on the Fig. 1, *e*, is due to changing the direction of burrowing on account of some obstacle.

The described typical mode of working is connected with many difficult problems.

1. There is the characteristic shape of the burrow to be explained. If we make a perpendicular hole in the sand about 5 cm. deep and drive the crab into it the animal soon starts working. But it never goes in the direction of the hole. The

burrow becomes bent and it approaches continually to the horizontal plane. This tendency explains to a certain extent why the burrow is never straight. The animal must hide itself in the sand and the shortest way of doing it would be the digging in a vertical direction. On the other hand the burrow must approach the horizontal line. Both tendencies working together cause the burrow to become bent. From a mechanical point of view we might expect that the crab will dig in the direction of a diagonal. Thus the burrow forming a curve shows that the causes are more numerous and a mechanical explanation is far too simple.

The biological significance of the end-chamber and of the burrow being bent follows from a simple experiment. When the animal has finished its work and it is sitting motionless in the end-chamber we begin to drop slowly sea water into the jar. Very soon the water reaches the crab. The animal begins to stir, then it climbs up to the mouth of the burrow and closes it. To do this *Uca*, usually sitting in the burrow with its large chela directed towards the entrance, goes entirely out, turns and enters the burrow with the chela I. forward. When the body is so deep that the bent legs 2–5 touch the edge of the hole they grasp this edge and pull it strongly inward. This time the legs are held close together and the leg 5 participates in the work like the others. There results a pellet of sand which is dragged down and adjusted carefully to the side wall of the canal close to its entrance. The pellet is followed by the second, third, etc., until a solid cork is formed closing tightly the hole. On the surface of the sand at the spot where the hole was, there remain radial furrows converging towards the former center of the hole. This process was already described by Pearse and others. But the task is not yet finished. *Uca* grasps the sand inside the burrow and carries it to the top, adjusting the pellet to the new formed roof of the hole. Very many pellets may be handled in the same way and a thick cork arises which closes the burrow tightly. We may pour so much water as to cover the sand completely. If at the beginning we dropped the water in very slowly the animal had enough time to close the burrow carefully. In this case, and such is the case under natural conditions, even under the water there remains always a waste chamber where air is retained and in which the crab remains until the tide goes out. This process may become modified in several ways. Very often *Uca* adjusts the sandcork in the middle of the canal without rising to the mouth of it. The end result remains the same, as the cork is also very tight. If we pour the water a little quicker the crab works in a great hurry. It rises to the top and it simply pulls many pellets of sand down without adjusting them. Sometimes the animal has no time to turn its large claw inward and it works hastily with II.–V. legs as well as it goes. Finally, if we pour the water very quickly, the burrow remains open, water enters into it, and the walls of the canal collapse burrying the animal. In such a case *Uca* usually very soon digs itself out and remains on the surface of the sand until we suck the water out with a pipette. In the field such an accident may be caused by a rain shower.

These observations pour some light on the biological significance of the burrow being bent and the end of it approaching the horizontal line. In such a burrow the end-chamber may be easier preserved from being filled with water. The air-chamber sometimes may be vertical as in the Fig. 1, *c*. But the foregoing part of the burrow is then strongly bent and the end result remains the same. Such air-chambers are known for several sand crabs, as for *Dotilla* (Symons, 19) and others.

Exactly speaking, the detecting of the biological significance of the shape of a burrow does not tell us anything about the factors that cause it. Beyond any doubt the direction of burrowing is closely connected with gravity. This follows from some experiments. In order to be able to watch closely the process of burrowing I constructed a simple apparatus. Two glass plates fitting closely into a jar in vertical position and reaching about two-thirds of its height were put into the vessel. Four corks of a suitable size (about 2 cm. thick) were put at four corners between the plates and the whole was held together with four threads. Thus the plates formed a kind of box and the 2 cm. wide space between them was filled with wet sand. Over this box standing vertically in the jar I put a round piece of cardboard fitting exactly to the jar and having a cleft in the middle corresponding to the space between the plates. If we put a crab into the apparatus it begins to dig and as it can dig only between the plates it may be watched during the whole work. When the crab reached about the half of the height of the sand column I turned the apparatus at right angles. *Uca* changed then the direction of digging, working now nearly at right angles to the previous direction. Some of such burrows may be seen in Fig. 3.

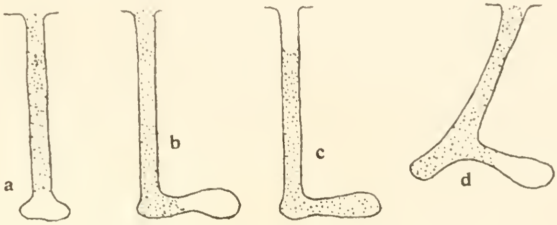

FIG. 3. Effect of turning the apparatus at 90° *a*, *b*, *c*, burrow of the same individual. For explanation see the text.

Clark (4) extirpated both inner antenna of *Uca pugilator*. He states that the normal equilibrium of the animal was somewhat damaged and there was a tendency to roll over. But at the same time the normal conditions, as feeding and burrowing, remained unaltered. Clark has not investigated the shape of the resulting burrows which could possibly furnish some interesting conclusions. For the moment we can say only that gravity is one of the factors affecting the shape of the burrow.

2. Another question connected with burrowing is the question of breathing. The interesting paper of Abbott dealing with the effect of distilled water upon the fiddler crabs (1) contains many important data. The gills of *Uca* are situated at the bottom of the large gill-chamber and the space over them is filled with liquid. Abbott thinks that this liquid has only about one-fifth of the concentration of the sea water. The gill-chamber communicates with the exterior by means of a canal, the opening of which lies between the basal joints of third and fourth legs. In this way the liquid of the chambers may exchange gas with the exterior. "In other words the crab when out of the water is able to breathe air" (1, p. 170). The invariable presence of an

air-chamber at the bottom of the burrow seems to support this view. It is probable in fact that the crab under its natural conditions practically is never out of the air. I kept several fiddlers in a jar the bottom of which was covered with a very thin layer of moist sand (2–3 mm.). The jar was covered and thus the atmosphere within it was saturated with vapor. The gill-chamber was prevented from drying but at the same time there was no chance of changing its liquid. Under such conditions the crabs lived very well for over 6 weeks and would probably have lived much longer had I not broken the experiment up. I failed to find anywhere data as to how long a fiddler may live under the water. Schwartz and Safir mention only that probably the fiddlers "do not find prolonged submergence very comfortable" (15, p. 19). And yet my fiddlers lived under the water for 6 weeks without showing any abnormalities and are still alive. To the end of this time they move rapidly when frightened and feed on mussels under the water. It is clearly to be seen that the flagella make their rhythmic movements, which in all crabs serve for renewing water in the gill-chamber. So the fiddler crabs seem to be true water-breathing forms like all other crabs. But at the same time the contents of their gill-chamber may be thoroughly ventilated in the air. It does not mean that they breathe air, nevertheless such a wide scale of adaptation furnishes an interesting example. The last experiment does not support the opinion of Abbott that the liquid of the gill-chambers has a lower osmotic pressure as compared with the sea water. It seems to me rather that this liquid cannot be anything else than the pure sea water.

3. A further question arises: why is the burrow of a fairly definite length, or, what conditions cause the crab to stop its working? There are several possibilities which may be tested experimentally.

The crab may stop after having expended a certain amount of energy in digging. But the resistance of the ground may be very different depending on the degree of moisture, presence of mud, small stones, plant roots, etc. The amount of spent energy may be very different in every single case and yet there is no evidence that the length of the burrow is affected by those factors. They bear undoubtedly only on the time of digging. In some experiments I put a glass ring at the periphery of the jar covering it with a layer of sand about 3 cm. high. The crab digging close by the wall soon reached the obstacle and for several minutes it tried to remove it. It abandoned then the burrow and began to dig another one in the neighborhood which it abandoned in turn. The animal made 5 successive holes until it succeeded in bending the canal towards the middle of the jar and in avoiding the obstacle. The total amount of expended energy was certainly much greater than in the usual case and yet the animal dug its hole to the very bottom of the jar. The whole process lasted about 6 hours, during which *Uca* was constantly working, while an ordinary burrow is made easily in half an hour. In another series of experiments I mixed the sand with at least the same quantity of small stones (about 6 mm. in diameter). The resistance of the ground was very much increased, in fact about 3 times as measured roughly by determining the weight which was necessary for driving a nail into the sand and into the mixture. Nevertheless the crab succeeded in making a regular hole reaching to the bottom of the jar, which lasted however for about 20 hours. The amount of energy is different depending on the degree of inclination of the canal towards the horizon, as carrying the sand out from a steep burrow requires a greater strain. And again this does not bear on the length of the burrows.

Another possibility is that the crab may estimate the total length of the burrow while carrying the sand pellets out. But one observation speaks against the validity of this factor. The mode of burrowing described above is a type which does not mean however that it is to be observed the most frequently. In very many cases the crab works in a slightly different manner. When the burrow is just deep enough to hide the animal, *Uca* comes out of it, turns its big claw towards the burrow and enters again. The legs II., III. and IV. grasp the sand from the bottom, but the pellet is not carried out of the hole. The legs push it towards the opening, while the body remains at the same spot. The pellet comes to lie between the sternum of the crab and the wall of the canal. Still pushing it with the external surface of the legs II., III., IV., the crab forces its own body below the pellet, grasping it with the legs 2, 3, 4. The sand is carried then to the mouth of the burrow and the legs 2, 3, 4, helped effectively by the small claw adjust the pellet at the edge of the hole. The second or the third pellet close the hole entirely, while the crab still continues the same work, grasping the sand at the bottom and adjusting it at the roof. As the work progresses the chamber containing the fiddler comes to lie still deeper under the surface and the sand-cork becomes still thicker. When the bottom of the jar is reached *Uca* spends a considerable time in working at the end-chamber and finally it quiets down, sometimes for many days. During the whole work the crab has no chance of measuring the length of the burrow as it is working itself through the sand. I think therefore that this factor may be also excluded.

If the importance of both mentioned factors is somewhat doubtful, it is sure that the degree of moisture plays a rôle in estimating the depth of the burrow. Even during the low tide I always found water at about one foot below the surface of the fiddler-ground. The burrow cannot go so far, for the walls of the end-chamber would collapse under the water. Testing the natural burrows with a long pipette I never found water there. The question may be solved experimentally. Usually I performed my experiments in the following way. I put sand into the jar and poured enough water to cover it completely. By stirring, droplets of air were removed. Then a long pipette was forced through the sand to the very bottom of the jar and the water was sucked out as far as possible. Under such conditions the crabs dug always to the bottom. But if we put the pipette only to half of the thickness of sand sucking water out, the animal will stop working when the level of water is reached. The degree of moisture influences then the depth of the burrow.

Unfortunately it is also sure that this factor is not the sole one. The natural burrows measured on an area of about one square meter on a perfectly flat ground may reach a very different level below the surface of the sand in spite of the exactly equal rising of the degree of moisture for all of them. Thus I am unable to answer exactly even such a simple question as why the burrow is of a definite length. One is sure that the behavior of a *Uca* is determined by very many different factors.

4. The question of periodicity in the life of the fiddler crab is a very complicated one, as there are some theoretical objections. In my opinion the problem of intrinsic periodicity of the life phenomena resembles very much the famous problem of the inheritance of acquired characters. The rising tide brings a variety of factors with it and so does the falling tide. The life of a crab is fairly periodic and this is due to an approximative periodicity of external factors. Now if we observe the animal in the laboratory where conditions are fairly constant, there are no factors which in nature cause the rhythmicity of behavior. How could we expect such a rhythmicity? Every kind of activity is a reaction to certain stimuli. The peculiar character of all these reactions is hereditary and fairly constant for each species. But it surely does not mean that in the entire absence of those stimuli the reactions would remain the same. Such a "memory" is an obvious impossibility. If the causes are absent the effect will be absent also. We do not think that under natural conditions the periodicity of behavior of a fiddler is due entirely to the "memory" and not to the periodicity of tides. But only in this case we could expect the animal to behave rhythmically under the constant laboratory conditions. As to the cause of all rhythmicity, the tides, it is interesting to note that they are not rhythmic at all. I quote a random instance from Eldridge's Tide-Book for 1925. Successive intervals between the high-tide, low-tide, high-tide, etc., during the ten days are, in minutes:

349
406
341
401
346
410
353
389
344
412
308
380
356
399
312
430
381
371
321
421
389
360
324
419
385
360
321
419
379
365
316
426
364
376
311
431
349
390
308
434

The lowest interval is 308, the highest 434, which corresponds or a difference of 2 hours 6 minutes. As such an irregularity tuns all the year round it would be a pure wonder if the "memory" would compel an animal living under constant conditions to accomplish its tasks at the same intervals. I do not intend to discuss the rich literature on periodicity of organic functions, but I have a strong impression that the whole question has a lot of metaphysics in it.

In fact I never noticed any intrinsic periodicity in my crabs. When put into the jar the crab digs the burrow, closes it and remains in the air-chamber for a very various time. Sometimes it will dig itself out in a few hours, sometimes it remains quiet for 3–4 days. If we put water into the jar while the chamber is tightly closed the crab does not go out even for a week. If we suck all water out *Uca* usually digs itself out in 3–4 hours, but sometimes it does not stir for many days. Its behavior must be ascribed to actual external factors, not to the remembered ones, although we are very far from understanding which factors are working. When the sand begins to dry *Uca* closes the burrow. It does the same when we pour slowly water into the jar and the sand becomes wet. From a certain point of view such reactions may be called memory, since the crab does the same as it has done under its natural conditions. We imitate those conditions as closely as possible and sometimes we get the same reaction. It is also not true that the fiddler closes its burrow "before the tide." It does it always *during the tide.* when the slowly rising water has moistened the air-chamber, not yet covering the surface of the ground. The arising slight movement of sand grains or the walls of the chamber becoming softer may be perceived by the crab as the beginning of the tide. As far as I observed *Uca* never remains a long time on the surface of the ground without visiting from time to time its burrow.

And thus, exactly as in heredity, not a given reaction in itself characterizes an animal, but always the faculty of producing this reaction *under given conditions.*

5. What becomes of the burrow after the tide? The tide destroys a part of the burrow and the crab must dig itself out. This may be done in several manners and I must confess that

the interpretation is very difficult. As mentioned above a quick pouring of water in the jar causes the walls to collapse, thus destroying the burrow completely. The animal forces itself through the sand and after the water has been removed it digs a new burrow in quite a new spot. But if the burrow was tightly closed *Uca* begins to work methodically, provided that the "tide" has gone out. Its movements are now somewhat the reverse of the ordinary burrowing. It will be remembered that the animal while remaining motionless in the air-chamber has its large chela directed to the inside. Now the outer legs, 2, 3, 4, grasp a sand pellet from the roof of the chamber and push it down towards the sagittal axis of the body. Then the crab climbs a little upward until the pellet lies between the carapace and the legs II., III., IV., which grasp it in turn. *Uca* comes down again and the sand will be deposited and kneaded at the bottom of the chamber. Still pursuing this work, the animal causes the chamber to rise slowly in an oblique direction, still keeping its volume unaltered, as the sand is always only carried from the roof of it to the bottom. At last the fiddler emerges, leaving the former canal filled with sand behind itself. Only the upper part of the burrow corresponding to the chamber in the last moment of work remains open. Now the crab may roam for a long time around the jar or it will start digging a new burrow at once. In most cases it works now along the same way, entering the hole from which it has emerged and following the same track. Usually it carries then the removed sand out of the burrow and the whole canal remains open. But, curiously enough, often will the crab close the canal behind it. After some time *Uca* is sitting again in the air-chamber at the bottom of the jar, while the whole burrow is tightly filled with sand, as if the animal wanted only to breathe fresh air for a while. In following the previous track the crab is evidently guided by the smaller resistance of the sand filling the burrow as compared with the surrounding. If the jar was filled with water for some time the sand collapses and there is no difference in its consistency. In this case *Uca* digs itself out once more, but does not follow exactly the previous track and sometimes it will dig in a perfectly vertical direction. In the apparatus described above, where the fiddler had to dig between two glass plates, this exact following of the former track may be easily observed. A female dug a vertical hole (Fig. 3, *a*) and then the whole apparatus was turned at right angles. The crab changed the direction of the digging (Fig. 3, *b*). All the time the burrow remained filled with sand save the end-chamber. On the following morning I found the female out of the apparatus and the hole showed conditions as on Fig. 3, *c*. The track of digging could be distinctly followed and it corresponds exactly to the previous one.

Nevertheless such a behavior is not a rule and I have seen many times that the crab may choose an entirely new direction. One individual has dug itself out and the burrow remained open for several hours. Then it entered into it, grasped sand from the bottom and carried it to the opening, closing it in the usual way. *Uca* then proceeded to work until the whole canal became tightly filled with sand. During the work the end-chamber increased both in volume and length, directing itself obliquely upward but at right angles to the former burrow. After having closed the burrow the crab immediately started again the work of digging itself out in the new direction. It detached pellets from the roof and deposited them at the inner corner of the chamber. After some time a new open burrow was manufactured and as the end-chamber has increased considerably during the previous work it had now room enough for all sand detached from its roof. As a result there was a new open burrow at right angles to the first. I am unable to say what may be the biological significance of such a peculiar behavior.

We see clearly at least that closing the burrow before high tide and opening it at low tide by no means covers the whole field of the behavior of a fiddler crab. It is merely a scheme under which lies a whole world of varied, complicated and as yet perfectly incomprehensible activities.

6. The most difficult problem, of course, is the problem of interpretation. I do not know how to characterize the behavior of *Uca*. Is it reflex, instinct, or intellect? It will be probably safer to speak about the "activities" and it is certainly more important to investigate which are the real properties of those

activities than to reason about what they are and how to call them.

There is one striking property of very many reactions which I would like to insist upon: their plasticity. In fact, the closer we watch a fiddler the more obvious becomes the conclusion that there is very little automatic in it. The practical mode of accomplishing the various tasks depends on an infinity of minute circumstances which are so various and different that surely never two crabs work at their burrows under exactly the same conditions. The animal performs its tasks *in spite of* the condition being so various, and yet there is no doubt that every single movement is largely depending on external stimuli. When the pellet is carried out, the sand must be prevented from falling back into the hole. And we see that in every single case the sand is pushed, pulled and kneaded *until* it becomes properly adjusted. Yet the properties of a pellet, as its size, its form, the degree of moisture, the contents of organic matter and of clay, the position towards the walls of the burrow, towards the mouth of it, towards the other pellets, etc., are never the same. All those properties bear very strongly on the actual movements of the animal, which has to adjust not simply "a pellet of sand" but always *this single pellet* with all its individual particularities. The crab is always so careful about it that only rarely a few grains roll back into the hole and it must estimate and judge whether the work is done sufficiently well. Every single activity connected with burrowing may be analyzed in the same way. Choosing the spot where the burrow is to be made, grasping the sand, carrying the pellet out, turning in the burrow so as to make it circular, taking care of the burrow being conveniently bent, conveniently oblique, of a definite length, avoiding the obstacles, manufacturing the chamber, closing the burrow in various manners, adapting the mode of acting to a situation which is different in every successive second, each of those activities again may be performed in infinitely different ways. But they all bear on burrowing, which is only a small part of the whole behavior of a fiddler crab.

Given this unlimited variety of reactions, it becomes utterly impossible to admit that each single reaction is referable to a particular fixed and unchangeable nervous mechanism. As may be deduced from the facts contained in the classical work of Bethe on the nervous system of *Carcinus*, this system is far too simple to be able to contain such a variety of reflex arcs. It does not follow from this that the activities may be controlled by something besides the nervous system. But it certainly follows from this that the morphology is of little help to us. We are compelled to admit a plasticity of the nervous centers; they must possess a certain creative power which enables them to become adapted to entirely new situations. The number of possible nervous connections is limited, but the number of possible reactions is infinite. This discrepancy may be avoided only by admitting that each nervous center may perform an infinity of functions.

SUMMARY.

The typical mode of digging a burrow is described and some problems discussed which are closely connected with the burrowing instinct.

The choice of the spot where the digging is started is determined by very many factors. Phototaxis and thigmotaxis are not sufficient explanations.

The end-chamber of the burrow functions as an air-chamber during the high tide.

The fiddler crab is a true water-breathing animal, but it can live in the air for several weeks without changing the water in the gill-chambers.

The length of the burrow partly depends on the degree of moisture of the ground.

There is no intrinsic periodicity in the life of *Uca*.

Several modes of closing the burrow and opening it are described.

In the interpretation the plasticity of activities is strongly insisted upon.

WOODS HOLE,
August, 1925.

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THE PARTHENOGENETIC EFFECT OF SPERM FILTRATES, CONCENTRATED SPERM SUSPENSIONS, AND SERUM OF CHITONS ON THE OVA OF THE SEA-URCHIN, *STRONGYLOCENTROTUS FRANCISCANUS*.[1]

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Kupelwieser (1906) reported that the unfertilized eggs of two species of sea-urchins, *Strongylocentrotus purpuratus* and *Strongylocentrotus franciscanus*, exposed to concentrated sperm or to filtrates prepared from heated sperm of two species (?) of chiton, formed fertilization membranes; and, if subsequently treated with Loeb's hypertonic sea-water, developed. Loeb (1907 and 1908) obtained similar results with the blood serum of different species of animals. In recent experiments I have obtained similar effects with the ova of *S. franciscanus*. In addition filtrates prepared from suspensions of living sperm of the chiton, *Katharina tunicata*, caused the formation of fertilization membranes in ova of *S. franciscanus*. Subsequent treatment with hypertonic sea-water led to the development of many of the ova in which membranes appeared.

These experiments were performed during the spring of 1921 at the Hopkins Marine Laboratory at Pacific Grove, California. I wish to express here my appreciation of the hospitality extended to me at this laboratory and my thanks to the Director, Dr. Walter K. Fisher, for his generous assistance.

MATERIAL.

Within a few miles of the Laboratory, in protected situations along the rocky coast, there is to be found an abundance of the sea-urchins, *S. franciscanus* and *S. purpuratus* and of the chitons, *Katharina tunicata*, *Ishnochiton magdalensis* and *Cryptochiton*, which were used in these experiments. All of these occur in similar situations within Monterey Bay. Where easy of access they are not as abundant, because they serve as food for the Japanese and the Italians of the region. *S. franciscanus* is to be found from a few to many feet below low water line and *S. purpuratus* just below this line. On calm days both species can be seen at depths of twenty feet and hundreds of feet from shore. *S. franciscanus* is rarely exposed at the lowest tides, whereas *S. purpuratus* is commonly found in isolated tide pools or completely exposed at low tide. In many locations this species is so abundant that the rocks are carpeted with this purple sea-urchin. The chitons adhere to the rocks by their pedal muscles (Sampson, 1894), and are exposed only at the lowest tides.

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[1] Contributions from the Zoölogical Laboratory of Smith College No. 137.

Ripe gametes of *S. purpuratus* were available from December until June and continued to be so, according to Dr. Fisher, until the middle of July in individuals from certain locations. In sheltered parts of Monterey Bay all individuals shed during the week of March 26, following a series of heavy rains. During the first week of May a similar shedding occurred at "Parke Davis Point," a sheltered location on the Pacific coast. In the most exposed collecting area, shedding had not occurred before the middle of July. Elder (1911) and Loeb (1915) have recorded similar breeding habits for this species. Few individuals of *S. franciscanus* contained fully developed gonads before April. The unusually cold season probably accounts for their slow development, as Dr. Frank Lillie had obtained plenty of ripe individuals in January and February of the preceding year in similar locations. In the chitons ripe spermatozoa were present in March, but ripe eggs were not obtained until the first week in May. In *K. tunicata*, according to a personal communication from Dr. Gertrude Van Wagenen, eggs were not shed until late in July in exposed locations. Heath (1899) states that the eggs of *Ishnochiton* are shed in May and June.

METHODS.

In the sea-urchins the oral disc was cut away; the contents of the body cavity, with the exception of the gonads, removed; and the body cavity thoroughly irrigated with filtered sea-water.[1]

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[1] All sea-water was obtained directly from Monterey Bay and filtered. Because

The gonads were transferred to Syracuse watch glasses, in which they shed their reproductive cells.[1] The gonads of the chitons lie just beneath the dorsal shells, and were exposed either by removing the latter or by removing the muscles and the viscera ventral to the gonads. The body cavity was thoroughly washed with filtered sea-water and the gonads placed in Syracuse watch glasses. The body fluid was obtained by piercing the pedal muscles of the chitons and allowing the body fluids to drain out into finger bowls. These fluids were then filtered through double layers of filter paper.

The filtrate of sperm suspension of *K. tunicata* was prepared by filtering ten per cent. sperm suspensions of this species through tested Mandler filters according to the method used in another investigation (Sampson, 1926).

RESULTS.

Exposure of the ova of *S. franciscanus* to a filtrate of a ten per cent. sperm suspension of *Katharina tunicata* for one to twenty minutes led to the formation of perfect fertilization membranes in the majority of the ova. If transferred to sea-water cytolysis occurred very rapidly in those eggs possessing membranes. If exposed for sixty to eighty minutes to hypertonic sea water many of those with membranes developed into gastrulæ and plutei which were normal in appearance.[2]

In highly concentrated sperm suspensions of *K. tunicata* the ova of *S. franciscanus* formed membranes within one to three minutes. From forty to sixty per cent. of the ova were so affected. Within ten minutes these membranes were perfect in contour. Dilute suspensions of spermatozoa were ineffective. The spermatozoa of this species are inactive in sea-water and in

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of copper in the pipes the running sea-water in the laboratory could not be used, nor could water be carried in galvanized iron pails. Enough zinc dissolved in the sea-water in transit to prevent the fertilization of eggs in it, or to prevent the fertilization of ova from females washed in such water.

[1] This proved to be the most satisfactory method of obtaining the reproductive cells from *S. franciscanus*, partly because of the large size of this sea-urchin and partly because the reproductive cells were so concentrated and so viscous that they did not shed readily through the genital pores.

[2] Ova of *Nereis* exposed to the sperm filtrate only of *Arbacia* formed membranes, maturated, and a small percentage of those with membranes segmented normally and developed into abnormal trochophores (Sampson, 1926).

egg-water of *S. franciscanus*, but are intensely activated in the actual presence of ova of the latter and form dense halos around them. Such eggs, freed from the excess of sperm by repeated washing in sea-water, fail to develop and those with membranes cytolyze in sea-water within twenty hours. If, however, the washed ova are subjected to a treatment with Loeb's hypertonic sea-water a certain percentage of those with membranes develop into gastrulæ and a smaller percentage into plutei. The best results were obtained when the ova were exposed to a concentrated sperm suspension for one and a half minutes and then to hypertonic sea-water for seventy minutes. At the end of sixty hours there were twenty-eight per cent. gastrulæ and seventeen per cent. plutei. The remainder had cytolyzed. The plutei lived for eleven days and the majority were normal in appearance. Similar results were obtained with concentrated sperm suspensions of *Ishnochiton magdalensis* and of *Cryptochiton*. Ova treated with hypertonic sea-water only for similar periods did not develop and such ova could subsequently be fertilized in sea-water with species-true sperm.

The blood serum of *K. tunicata* produced results similar to those obtained with sperm filtrates or with the concentrated sperm suspensions of this species. If concentrated serum is used the resulting membranes are wrinkled and unevenly elevated. If the serum is diluted twenty to thirty times normal membranes are produced. (Robertson (1912) obtained his best results with diluted serum in experiments with ox blood on ova of *S. purpuratus*.) The subsequent cytolysis in sea-water can be prevented and the development of many of those with membranes obtained if the ova are treated with hypertonic sea-water for seventy minutes after exposure to the serum. Similar results were obtained with dilutions of the serum of *Ishnochiton magdalensis* and of *Cryptochiton*.

The ova of *S. purpuratus* were used in similar series of experiments. In place of a typical membrane, ova exposed to test preparations, appeared to be surrounded by a swollen transparent jelly which tended to dissolve in sea-water. Such eggs could be fertilized in sea-water with sperm of the same species at once or two hours later. Such eggs if treated with hypertonic sea-water did not form membranes or develop.

CONCLUSIONS.

Ova of *S. franciscanus* exposed to sperm filtrates, concentrated sperm suspensions, or to blood serum of *Katharina tunicata* for one to three minutes formed typical fertilization membranes. Subsequent treatment with Loeb's hypertonic sea-water for seventy minutes caused a large percentage of those with membranes to develop into gastrulæ and plutei. Every precaution was taken to avoid contamination of the sperm suspensions of the chiton with body fluids or serum. It seems probable that the membrane formation in the filtrates and in the concentrated sperm suspensions is formed by some substance derived from the sperm.[1] The nature of this substance has not been determined. Experiments with filtrates of sperm of *Arbacia*, *S. purpuratus* and *S. franciscanus* and *Nereis* indicate that it is an organic compound (Sampson, 1926). The effect produced by the serum is probably due to organic compounds present in the latter.

Similar results were obtained with ova of *S. franciscanus* which were treated with concentrated sperm suspensions and with dilute serum of two other chitons, *Ishnochiton magdalensis* and *Cryptochiton.* With ova of *S. purpuratus* no typical membranes formed in the preparations used, and the fertilization capacity of the treated eggs appeared to be normal.

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[1] A membrane swelling has been observed in concentrated suspensions of sperm by Heilbrunn (BIOL. BULL., 29, p. 149–203), and attributed by him to a substance produced by sperm.

VASODILATATION IN *FUNDULUS* DUE TO A COLOR STIMULUS.

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In a previous issue of the Biological Bulletin,[1] the writer gave an account of the results of experiments with differently colored environments on *Fundulus*. These experiments were carried out in the Harvard Zoölogical Laboratory during the winter of 1922–1923. It was stated in that article, that the distinctive coloration obtained when *Fundulus* was placed in a red environment was due to a vasodilatation. It was further stated in a postscript that similar experiments were carried out elsewhere by the writer during the summer of 1924 with the same adaptive color changes occurring, but that no vasodilatation was observed when *Fundulus* was placed in a red background. It was suggested that the different results might be due to seasonal or local differences in the fishes.

During the past summer further tests were made at the U. S. Bureau of Fisheries Laboratory, Woods Hole, to determine the factors causing this difference. Positive results were obtained which will be briefly reported here.

In these experiments the fishes were first made to assume the light shade by being placed on a white background and from this stock they were selected for the various tests. Five specimens were placed in each of four battery jars containing fresh water and the jars placed in the differently colored boxes. Adaptation to yellow, blue, green, and red backgrounds were obtained with a vasodilatation occurring in specimens from the red background.

The experiments were repeated under the same conditions except that salt water was used. Adaptation to the various colored backgrounds occurred, but vasodilatation was not observed in the specimens placed in the red environment at the same period of exposure to the stimulus.

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[1] Adaptive Changes in Shades and Color of *Fundulus*, Biol. Bull., Vol. XLVIII., January, 1925.

To make a more precise comparison of the effects of a red environment, two tests were made at the same time under identical conditions except that in one set fresh water was used, in the other, salt water. When both sets were compared on a white surface on the third day, there was a decided difference in the appearance of the fishes, those in the salt water lacking the striking pink coloration. There was, of course, a general similarity in coloration, due to definite states of contraction of the chromatophores whether in salt or fresh water, but the pink condition due to vasodilatation was absent in the former case. There was however a slight vasodilatation even in the specimens placed in salt water, especially under a prolonged stimulus, yet not sufficient to give the distinctive pink coloration of specimens placed in fresh water. The salt water must therefore inhibit, though not completely, the vasodilatation.

The rapidity with which this vasodilatation occurs varies with the age and vigor of the fishes. In young vigorous specimens from 6 to 9 cm. in length, placed in fresh water it never fails to occur, generally on the second or third day of exposure to the red background, though it is frequently intensified by the fifth day. An adaptive change due to the movements of the chromatophores occurs much earlier being already noticeable in a few hours.

If the specimens from the red environment are immediately placed on a white surface in good light, the condition of vasodilatation can readily be observed. It is most noticeable about the head, especially in the two large vessels of the dorsal region; also at the end of the spinal column and the base of the fins. The peripheral vessels along the dorsal median axis of the trunk are also strikingly dilated. It is interesting to observe here, the rapid disappearance of the dilation. If one fixes the attention on definite peripheral vessels, the change will be noticeable within one minute, and in about two minutes the phenomenon of vasodilatation largely disappears.

A close approximation to the condition produced by the red environment in *Fundulus* was obtained with the use of adrenalin. The fishes were first made to assume the very dark shade by being placed on a black background until their melanophores were maximally expanded. An intra-peritoneal injection of 0.50 to 0.75 cc. of adrenalin chloride—dilution 1 to 1000—was then given. In about 30 seconds a contraction of the melanophores was noticeable and this contraction, as judged by the unaided eye, was completed in about two minutes, when the fish assumed a pale grayish hue. In two to three minutes it began to assume a yellowish color, due to the partially contracted xanthophores in which a dense orange concentration of pigment might be seen on microscopic examination. In three to four minutes, a dilatation of blood vessels was noticeable at the base of the fins, especially the dorsal fin and about the head. The dilatation extended over the body generally within five or six minutes. In about twelve to fifteen minutes the coloration was similar to that obtained under the influence of a red environment, except that the adrenalin caused a more pronounced dilatation in specific regions as for example in the fins. The yellow color gradually diminished, but the vasodilatation persisted for hours. In one specimen the maximum degree of dilatation was observed five hours after the injection of adrenalin. The time periods for the different phases of the reaction varied slightly according to size and vigor of the fishes and the amount of adrenalin chloride injected. But those given above are typical of a dozen experiments on specimens 7 to 8 cm. in length.

If the caudal fin be examined under the microscope when the injection is made, it will be seen that there is an immediate constriction of the blood vessels causing a stoppage of circulation. This is soon followed by a renewal of the circulation which gradually extends over the fin. Adrenalin causes an immediate constriction and a post-dilatation of the blood vessels in *Fundulus* as in human beings. Apart from giving a convenient control for experimentation this post-dilatation effect of adrenalin is itself an interesting phenomenon in the light of a similar effect produced by a red environment.

SECRETION IN THE AMITOTIC CELLS OF THE CRICKET EGG FOLLICLE.

MARGARET R. MURRAY.

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I. INTRODUCTION.

The investigation of the cricket follicle-cell was undertaken primarily with the aim of observing its method of division (described by E. G. Conklin in 1903 as a type of the amitosis of senescence). Interpretation of the nuclear phenomena was, however, so greatly modified by study of the cytoplasmic phenomena paralleling them, that the nuclear division came to be considered rather as incidental to the other activities of the cell than as a manifestation important in itself. For the large amount of yolky substance with which the cytosome of the ovarian egg is provided, is elaborated through the agency of the follicle cells. These assume intense secretory activity during the later stages of the oöcyte's development, a time coincident with their own period of amitotic division. This paper contains an account of the nuclear and cytoplasmic phenomena of the amitotic cells of the cricket egg-follicle, observed in specimens living and fixed, during their period of secretory activity.

The investigation was carried out for the most part at Washington University, Saint Louis, in 1923 and 1924 under the direction of Professor Caswell Grave. Additions and revisions were made at the Hull Zoölogical Laboratory of the University of Chicago in the fall of 1924, by the courtesy of Professor F. R. Lillie. It is a pleasure to take this opportunity of expressing my thanks to Dr. Grave for valuable suggestions and criticisms; and to Dr. Margaret Reed Lewis, for suggestions relating to the technique of tissue cultures.

II. MATERIAL.

The cricket ovary consists of a bundle of egg-tubes situated in the posterior part of the abdomen, ventro-lateral to the digestive tract, and communicating by means of a short oviduct with the vagina. Each egg-tube contains a number of eggs (usually from 8 to 12 at a time), arranged in linear series according to size; the largest and most mature egg being adjacent to the oviduct, and the smallest located at the opposite end of the egg-tube, connected distally with a "nest" of undifferentiated cells. (See Fig. 15.) The egg-tube, or follicle (which descends with the egg), is one layer of cells in thickness. Between the egg and its follicle a rather thick vitelline membrane intervenes which exhibits hexagonal markings that correspond with the outlines of the bases of the superimposed follicle cells. The follicle cells themselves are of an epithelial nature, and approximate hexagonal prisms in shape, varying in proportions with age (to be discussed more fully further on) from the columnar to the squamous type. Except at the proximal end, where it is attached to the oviduct, the egg tube lies within the hæmocoel of the insect; but contact with the blood is prevented by a thin, closely investing membrane.

The species of cricket used for study were, *Gryllus abbreviatus*, the rather large, short-winged field-cricket; and *Nemobius fasciatus*, the small, striped ground-cricket. In practically all of this work, the two crickets could be used interchangeably, since the only differences between them so far as the ovarian tissue is concerned, involve the size of the egg-string as a whole, and the number of cells constituting an egg-follicle.

Since, in the history of any one egg, the stages designated in Fig. 15 as *A*, *B*, *C*, etc., form a continuous series, there is of necessity considerable overlapping between the conditions described for any two contiguous stages. In general, however, rapid and copious secretory activity is observed in the *A* and *B* follicles and to some extent also in the *C* follicle during September and October. Mitotic figures have been observed in cells of follicles distal to the *C* egg; only amitotic nuclear changes appear in stages *A*, *B*, and *C*.

III. METHOD.

The follicle cells were studied in tissue cultures with and without the use of vital stains. They were also studied after treatment with six fixatives and twelve stains, with their various possible combinations.

The culture medium most successfully used is a Locke solution as modified by Lewis and Robertson (M. R. Lewis and W. B. Robertson, '16):

Sea water	30 cc.
Distilled water	50 cc.
$NaHCO_3$	0.02 gram
Dextrose	0.25 gram

To this was added 0.1 gram of peptone. The tissue was placed in a hanging drop with a cover slip as growth support. In general, the whole egg string distal to the *A* egg was used in one implantation; the *A* egg was usually implanted separately. In spite of precautions against septic conditions, the cultures had an unusual tendency to become infected, especially with coccus and bacillus forms. The greater number of the cases of infection were probably due to the difficulty, at the time of implantation, of keeping the ovary and the dissecting instruments from contact with the contents of the digestive tract.

Healthy follicle cells have a very characteristic refraction and lie regularly arranged in the epithelial layer, as hexagonal prisms with slightly rounded corners. Dying or dead follicle cells exhibit a marked decrease in refractive properties, a more opaque appearance, and a loss of their regular arrangement, due usually to plasmolysis. In general, the cells remain in healthy condition from 48 to 72 hours after implantation.

The vital stains used were methyl green, neutral red, toluidine blue, brilliant cresyl blue, nile blue sulphate, and janus green, dissolved in the culture medium in concentrations varying from 1 in 20,000 to 1 in 5,000. The stains were toxic in greater or less degree; janus green, toluidine blue and nile blue sulphate were especially so.

The fixatives used were as follows: Bouin's; Flemming's (strong formula, with acetic); Da Fano's cobalt nitrate modification of Cajal's method for Golgi apparatus (C. Da Fano, '20); Regaud's fixative for mitochondria; formol; Kopsch's osmic acid impregnation for Golgi apparatus; and Champy's fluid modified for showing Golgi apparatus. For stains and combinations of stains with fixatives, see Charts I., II., and III. The fixatives served as controls for one another. The egg string distal to the *A* egg was sectioned longitudinally. The *A* egg could not successfully be sectioned longitudinally (for use in cytological study) due to the brittleness of the fixed yolk. Transverse sections were made of the *A* and *B* eggs. Sections varied in thickness from 2 micra (in material fixed by Regaud's method) to 5 micra (after fixation in Bouin's solution).

IV. OBSERVATIONS.

A. Cell Division.

Follicles were observed in culture medium for nuclear division. Specimens of the "amitotic" follicle fixed in Bouin's solution show what appears superficially to be a mechanism which might function in nuclear division, *i.e.*, a series of strands radiating from the plasmosome, with particles of chromatin often resting upon them (Fig. 2). It was expected that a study of tissue cultures might throw light upon these structures. To this end, follicle cells whose nuclei were in an elongated or constricted

COLOR REACTIONS OF *A* AND *B* FOLLICLE CELLS OF THE CRICKET, WITH VARIOUS FIXATIVES AND STAINS.

Fixative and Stain.	Mitochondrial Granules.		Small Yolk Globules.	Refractive Proximal Granules in Cytoplasm.	Large Yolk Globules.	Intermediate Yolk Glob.	Distal Droplets.		Nucleolus.	Chromatin Particles.
	In Nucleus.	In Cytoplasm.					Sheath.	Core.		
Regaud: $K_2Cr_3O_7$, Acid Fuchsin........	—	Red	Red	—	Red	—	Red	—	Red	Dark-red
Acid Fuchsin, Methyl Green....	—	Red	Red	—	Green	—	Red	—	Pink	Dark green
Iron hæmotoxylin.............	—	Black	Black	—	Black	—	Black	—	Gray	Black
Sudan III (alcoholic solution)...	—	Deep orange	Deep orange	—	Pale-orange	—	Deep-orange	Orange	Clear-greenish	Clear-greenish
Nile blue sulfate..............	—	Lavender	Lavender	—	Blue	—	Purple	—	Blue	Dark-blue
Erlich-Biondi.................	—	Red-orange	Red-orange	—	Red-orange	—	Red	—	Red-orange	Red-orange
Da Fano: AuCl, $NaHSO_3$...............	Black	Black (some annular)	Brown→ black (some annular)	—	—	—	Black	—	—	—
AuCl, $NaHSO_3$, Delafield's hæm.	Black	Black	Brown→ black	—	Lavender	—	Black	—	Lavender	Lavender
Nile blue sulfate..............	Black	Black	Brown→ black	—	Blue	—	Black	Pale-blue	Blue	Blue
Sudan III (alcoholic sol.).......	Black	Black	Brown→ black	—	Pale-yellow	Pale-yellow	Black	Orange	Clear-greenish	Clear-greenish

Color Reactions with Various Fixatives and Stains.

Fixative and Stain.	Mitochondrial Granules.		Small Yolk Globules.	Refractive Proximal Granules in Cytoplasm.	Large Yolk Globules.	Intermediate Yolk Glob.	Distal Droplets.		Nucleolus.	Chromatin Particles.
	In Nucleus.	In Cytoplasm.					Sheath.	Core.		
Da Fano: Erlich-Biondi	Black	Black (some annular)	Brown→black (some annular)		Red-orange		Black			
Osmic impregnation: Unstained	Black	Black	Black		Brown	Brown→ black	Black (present?)	Black	Greenish-yellow (black outline)	Greenish-yellow (outline of black granules)
Delafield's Hæmotoxylin	Black	Black	Black		Brown	Brown→ black	Black (present?)	Black	Brown	Brown
Delafield's Hæm., Methyl green	Black	Black	Black		Brownish-green	Black	Black (present?)	Black	Greenish-brown	Greenish-brown
Formol: Scharlach R (alc. sol.)	Orange		Orange			Orange	?	Orange		
Champy: Unstained	Black		Black	Golden yellow	Light-brown	Black	?	Black	Greenish	Greenish
Delafield's hæm., Methyl green	Black		Black	?	Dark-brown	Black	?	Black	Greenish	Greenish
Iron hæmotoxylin	Black		Black	?	Dark-brown	Black	?	Black	Brown	Black
Flemming: Iron hæmotoxylin					Black		?		Black	Black
Bouin: Delafield's acid hæm.			Rose	Pale rose	Reddish-brown		Reddish-brown		Red	Red
Iron hæmotoxylin, Eosin			Purplish-rose		Rose→black		Black		Black	Black
Erlich's hæmotoxylin					Purplish-rose		Dark-purple		Light-purple	Dark-purple

Color Reactions with Various Fixatives and Stains.

Fixative and Stain.	Mitochondrial Granules.		Small Yolk Granules.	Refractive Proximal Granules in Cytoplasm.	Large Yolk Globules.	Intermediate Yolk Glob.	Distal Droplets.		Nucleolus.	Chromatin Particles.
	In Nucleus.	In Cytoplasm.					Sheath.	Core.		
Bouin:										
Gentian violet, Gram's solution............	—	—	Red	Pink	Pink→purple	—	Red	—	Red	Red
Gentian violet, safranin.	—	—	Red	Pink	Rose	—	Red	—	Red	Red
Safranin, Licht-grün....	—	—	Dark-red	Pale-pink	Rose→green	—	Dark-red	—	Bright-rose	Dark-red
Erlich-Biondi..........	—	—			Red-orange	—	Red	—	Red-orange	Red-orange
Sudan III. (alc. sol.)....	—	—			Orange	—	Dark-orange	—	Greenish-yellow	Greenish-yellow
Nile blue sulfate......	—	—			Blue	—	Bluish-green	—	Blue	Green
Tissue cultures:										
Janus green..........		Deep green					Pale-green	—	Green outline	
Methyl green.........									Green	Green
Brilliant cresyl blue...		Blue?								
Neutral red.........							Red?	Red		
Toluidine blue..							Blue	—		
Nile blue sulfate ...			Purplish-blue		Pale-red	Bright-red	Deep-lavender	Pale-red		

state at the time of implantation were drawn with camera lucida and observed from time to time for periods of which the maximum was five days; but no significant change in shape of either nucleus or nucleolus was noted in either stained or unstained cultures, although the procedure was repeated many times. It is possible that the nuclear strands may have changed slightly in relative position, but such a change was difficult to observe with accuracy. The cells of the *D* and younger follicles were found too small to be observed satisfactorily.

When it was ascertained that division could not be seen taking place in the cells of the *A* and *B* follicles, forty follicles of the large cricket were fixed and sectioned. Of this group, ten *A* follicles were sectioned longitudinally and ten transversely; and the number of cells in the largest section of each longitudinally-cut follicle was counted, as well as the number of cells in the largest section of each transversely-cut follicle. The same procedure was carried out for twenty *B* follicles. Results follow:

Fol. No.	*A* Egg. No. Cells in Largest Circum.		*B* Egg. No. Cells in Largest Circum.	
	Long. Sect.	Trans. Sect.	Long. Sect.	Trans. Sect.
1	192	43	220	68
2	186	62	166	69
3	180	67	232	64
4	180	62	204	68
5	190	68	184	60
6	200	76	200	60
7	184	72	238	59
8	202	65	174	62
9	196	61	220	58
10	190	48	140	70
Total	1,900	624	1,978	660
Average	190	62.4	198	66

From the foregoing tables it appears that while there is considerable variation among individual follicles of each class as to their number of cells, the average number in the *B* follicle is similar to the average number of cells in the *A* follicle, although there is great difference in size between *A* and *B*. This indicates that during these two stages very little, if any, increase in the number of cells in the follicle occurs. Moreover, nearly all of the nuclei in these stages, as well as in the *C* stage, are very much elongated and distinctly constricted, and contain two or sometimes more nucleoli (Figs. 1, 2, 4); but this elongation, when found in the *B* or *C* follicles, is always in a plane *perpendicular* to the surface of the follicle, so that the constriction if completed would divide the follicle into two layers, one beneath the other. In no follicle has such a state of delamination been observed; but when the follicle is approaching the *A* stage, many of the nuclei appear to have oriented themselves so that their axis of elongation is *parallel* to the follicular surface. Nuclei which are apparently in transition from one position to the other may be seen rather frequently (Fig. 8). Coincident with this shift in the position of the nucleus, a change in the shape of the follicle cell from a columnar to a squamous form takes place; so that from being elongated like the nucleus in a plane *perpendicular* to the follicular surface, the cell becomes expanded and flattened in a plane *parallel* to that surface, growing in absolute volume the while. This fact seems to account for an enormous increase in the surface area of the follicle as a whole, between the *B* and the *A* stages (occupying from one to two months), without cell division. The results of the tissue cultures indicate that the elongation and constriction of the nucleus and the division of the nucleolus is at most an extremely slow process. (Conklin, '03, suggests that frequent division takes place between the *D* and *B* stages, and that division is very slow in the *A* stage.)

B. METAPLASMIC INCLUSIONS OF FOLLICLE CELL AND EGG.

1. *Distal Droplets.*

Unstained follicle cells in culture, used as controls, show compact and very highly refractive nucleoli, somewhat less refractive chromatin particles, and slightly refractive nuclear strands. The cytoplasm has a rather homogeneous and finely granular appearance, except for a number of large and exceedingly refractive granules which are usually present and, whenever found, appear in the part of the cell distal to the nucleus (Figs. 7, 8, 13). There is some, not a great deal, of variation from cell to cell in the numbers of these droplets, but in general they are most numerous in the cells of the *B* stage of the egg tube. Within any given cell the distal droplets vary in size in a rather regular manner, the smallest being nearest to the nucleus,—often applied to its membrane, the largest being adjacent to the distal cell wall. When viewed from above, they often appear to form a ring just above the nucleus (Figs. 11, 12). This position, and the fact of their gradation in size seems to indicate a close relationship with the nucleus at the time of their origin.

In the living cell these droplets stain red all the way through with neutral red. But with toluidine blue and with janus green only the outer rim takes on color. Nile blue sulphate has been demonstrated by J. L. Smith to stain fats differentially; *i.e.*, neutral fats are stained red, and fatty acids blue, the degree of acidity being indicated roughly by shades of lavender. When the egg string is stained with nile blue sulphate, the distal droplets show a pale red medulla with a deep lavender cortex. The droplets stain as a whole with scharlach R after fixation in formol; they are blackened after impregnation with osmic acid, and after treatment with Champy's mixture of osmic and bichromate. When tissues fixed with osmic acid are later treated with turpentine, the medulla dissolves first, leaving an indistinct gray base; the cortex dissolves only after long exposure. After other treatments, such as Regaud's, Bouin's, and Da Fano's, the medullary portion is dissolved out in the alcohols, leaving only a thin base which will take certain stains (see charts); but the cortical portion is preserved, usually as a ring of very small droplets.

The distal droplets were observed for indications of movement, at various periods after the explantation of the egg string in unstained media and in media stained with nile blue, toluidine blue and methyl green respectively. Movement of the distal granules was found to occur at all periods, and to occur so rapidly during the first four or six hours after implantation, as to be almost perceptible. These droplets, which were congregated in the distal region of the follicle cell, were observed with the low power to pass through the external membrane of the egg string. Shortly after implantation, the passage occupied on the average about three minutes; *i.e.*, three minutes were consumed between the time when the first suggestion of a droplet appeared on the outer surface of the external follicular membrane, and the time when the exuding droplet became perfectly spherical and separated itself from the membrane and lay free in the culture medium. The fact that the droplets pass out so readily indicates that the membrane is coarsely porous. It should be added that the cultures observed for the movement of the distal granules were uninfected and in good condition otherwise, hence the activity recorded is presumably a typical one, and not a result of fatty disintegration of the follicle cells. Moreover, the number of distal droplets in the cells does not tend to increase with the age of the culture, but rather to decrease. This decrease is coincident with the gradual decrease in the food and oxygen supply afforded by the medium.

By means of fixation and of vital staining, a histo-chemical identification of these cell inclusions is somewhat dubious. There may, however, be some value in suggestions as to what class of compounds the various inclusions belong. To this end the Table of Chemical Constitution of Fatty Substances (by Dr. W. Cramer in Lee's "Vademecum," page 358), Kingsbury ('11), Bell ('14), Bowen ('19), Smith and Mair ('10), and other works have been consulted with reference to the charts of the color reactions of these inclusions. The constitution of the medulla of the distal droplets seems to be something in the nature of a true fat, mixed, or perhaps combined, with a little protein, as suggested by the grayish base left after treatment with solvents. All the reactions of the medulla except its ready solution in turpentine after osmic acid fixation might be interpreted as the reactions of Golgi bodies. The latter, however, relegates it to some other group of lipoids, as does its passage into the hæmocoel. The cortex reacts as a lipoid with the lipoid fixatives, but is preserved through the alcohols after treatment with Bouin's fluid.

As to the function of the distal droplets in the life of the organism, I have as yet little or no indication. It is possible to conceive of them as an endocrine secretion from the follicle, although this seems unlikely in the insects; or, they might be merely excretory products.

2. *Mitochondria.*

In addition to the distal droplets, the cytoplasm of the follicle cell contains usually large numbers of small spherical granules, which are most numerous in the proximal region, but are found distally also. There is always a row of them applied to the nuclear membrane. These granules I shall speak of as mitochondria, since they conform in general to current descriptions of the form and distribution of cell inclusions to which that name is given, and since they stain red with acid fuchsin or black with hæmotoxylin after the Regaud treatment (Fig. 1), and stain with janus green in cultures (Figs. 9, 10). They are blackened with osmic acid (Fig. 5), and with silver nitrate (Fig. 4). Bouin's, Flemming's (acetic), and Champy's treatments do not preserve them (Figs. 2 and 3). Their numbers conform with the observations of Cowdry that abundant mitochondria are associated with intense protoplasmic activity. If such a relationship be granted, there are in these tissues indications of waves of protoplasmic activity: in many follicles, notably those impregnated with osmic acid, groups of cells which show a large number of mitochondria are contiguous with groups showing a lesser number, and these in turn with groups showing more, etc. Since the egg tubes are quite discrete, there can be no question of differential penetration of the fixative.

These mitochondrial granules are continuous in position, however, with granules in both the yolk and the nucleus of the follicle cell. In these three places they seem to be *generally* similar in lipoid nature as detected by staining reactions, but not *exactly* similar (see charts). In many cases, suggestively oriented rows of them can be seen, some members of which are in the vitelline membrane, and others, exactly similar in appearance, are beyond the membrane, within the egg (Fig. 1). Beginning a short distance within the vitelline membrane, they are found characteristically grouped around the other yolk constituents. In the Da Fano preparations, granules can be seen in the plasmosome of the follicle cell, and also in rows along the nuclear strands which radiate from it (Fig. 4). This is reminiscent of Saguchi's findings in the frog pancreas. It also suggests that the nuclear strands in the follicle cells may have a metabolic significance. Essentially the same appearance is produced by impregnation with osmic acid (Figs. 5, 6): granules are not seen *within* the plasmosome, but form a sharp cortical sheath around it. In Champy preparations (Fig. 3), black granules are seen on the plasmosome, as well as surrounding it. (Here the plasmosome also contains clear yellow granules, somewhat larger than the black ones.) With scharlach R after formol, granules sheathing the nucleolus and scattered in the nucleus stain orange.

These observations seem to indicate a sequence or metamorphosis of granules from nucleolus of follicle cell to yolk of egg, with the mitochondrial state as one stage in the sequence. When they have reached the egg cytosome, the granules probably affect in some way the condition, or perhaps the synthesis of the other types of yolk globules, which they invariably surround. (Cf. Gatenby on Insect Ovogenesis.) It would, of course, be very desirable to see this process going on in the tissue cultures. This was attempted without success. It seems possible to attribute the failure partly to the opacity of the follicle cells (it was impossible to use either camera lucida or projection microscope on the small granules in order to detect motion over a long period of time); and partly to the extreme slowness with which the process must take place (August to November to produce the two to three cubic milimeters of yolk which are found in a mature egg).

3. *Yolk Globules.*

The arrangement of yolky materials in the oöcyte is a very definite one, as illustrated by the figures, especially Fig. 1, which represents an area extending from the external follicular membrane to the longitudinal axis of the egg. The yolk globules are oriented in the pattern indicated, with reference to the *periphery of the egg*, that is, with reference to the follicle cells. The nucleus of the egg is often distinctly eccentric, but that does not alter the arrangement of the yolk with reference to the follicle. Besides the small mitochondrial, or fuchsinophile, granules already referred to, the egg cytosome contains two types of globules. One of these, which will be referred to as the "large" type of globule, is at its maximum size a short distance from the vitelline membrane in a moderately ripe egg (Fig. 1); in a thoroughly ripe egg the large globules are nearly all of the same size. The large type of globule appears red after fixation by Regaud's formula and stains with acid fuchsin. If methyl green is then applied, the red color is lost, and a green color taken on. Nile blue sulphate after silver nitrate impregnation stains these globules. They are colored a dark brown by osmic acid, stained a pale red by nile blue sulphate (in culture), and left unstained by scharlach R after formol. They are colored a very light brown by the Champy treatment. They are preserved by Bouin's and Flemming's fixing fluids containing acetic acid. It is suggested that these globules may be of a constitution on the order of lecithin.

There is some indication from tissues fixed in Bouin's and Champy's fluids, of the origin of the large type of yolk globule. Clear granules can be seen in the proximal part of the follicle cell, often forming rows from nucleus to cell membrane, and continuous with an increasing series of this type of yolk globule (Figs. 2, 3). The staining reactions inside, and outside, the cell membrane, are somewhat different. In the yolk mass these globules are preserved and stained with great readiness; the globules in the follicle cell take little color from the ordinary stains.

The other type of yolk globule, called "intermediate" in size, and represented in Figs. 1–4, is distributed in much the same pattern as the "large" type, but does not have as great a size-maximum. It is dissolved by either fixative or alcohols during all the treatments except Kopsch's and Champy's (in which it is blackened), and formol (after which scharlach R stains it a deep orange). Nile blue sulphate, used in the cultures, stains it a bright red. Turpentine dissolves this type of globule after osmic acid impregnation. It is probably, therefore, very close to a true fat in composition. The intermediate type of globule is always found surrounded by a fuchsinophile cortex—usually granular; but no evidence of a direct origin from the follicle cells has been observed for it, although it is evident that the materials of which the globule is synthesized must, like the others, reach the egg by way of the follicle cells. In the proximal region of some of these cells there has been seen a nebulous structure composed of very finely divided black particles, which might be considered to indicate an excessive deposition of fat in the tissue. This fine emulsion might conceivably be coarsened at the boundary of the cell, and coalesce into fatty globules. (See Fig. 5.)

What part the egg itself may play in the synthesis of these yolky materials has not been ascertained. It is remarkable that no substances which could be identified as Golgi bodies have been found in either follicle cell or egg, although several standard fixatives for Golgi bodies have been used, and many kinds of inclusions preserved. With the use of either the Champy or the Kopsch preparation alone, doubtless the distal droplets would have been considered to be Golgi bodies. But all the observations combined make such a diagnosis doubtful for these or any other observed inclusions, in either egg or follicle.

V. DISCUSSION.

A. Significance of Nuclear Constriction.

A general discussion of the significance of amitosis and a review of the literature on the subject will not be attempted here in view of the full surveys afforded by Conklin (1917), Nakahara (1918), and Bast (1921).

Study of cell division in the "amitotic" follicle cells of the cricket has shown that in this form the division of the nucleolus and constriction of the nucleus rarely if ever are followed by the constriction and division of the cytoplasm; it has also shown that complete cell division is not necessary to account for the increase in the size of the follicle between the *B* and the *A* stages. That the amitotic behavior of the nucleus here is an indication or result of senescence seems open to question in view of the fact that at this time the follicle cells are metabolizing at a high rate in the elaboration of materials which go to make up the yolk of the egg, and therefore cannot, so far as metabolism is concerned, be called senescent. They are, it is true, highly differentiated cells, and in that sense the term senescent may be applied to them. But it seems to the writer that this amitotic behavior, confined as it is to the nucleus, should be given a more particular interpretation, when it is found in connection with intense secretory activity, in which the nucleus plays so large a part as in these follicle cells. The proportionately great increase in nuclear and nucleolar surface which is the result of amitosis in these cells would appear to be a distinct advantage in their secretory activities, and might represent one phase in the cells' differentiation. Indeed the behavior of these cells bears little resemblance to the usual processes of cell division.

B. RELATIONSHIP OF MITOCHONDRIA WITH YOLK.

It is not generally conceded by American cytologists that mitochondria actually metamorphose into the constituents of yolk, or of other secretions; nor is it even generally held that these bodies play an intimate part in the production of secretions by secretory cells. Lewis and Lewis (1915), in a review of the literature on mitochondria and a description of their own observations of mitochondria in tissue cultures, state that they find no direct relation between mitochondria and the formation of fat. In a summary (1916) of the functional significance of mitochondria, Cowdry quotes Mathews (1915) in saying that mitochondria are related to the phospholipins found in all cells, whose function undoubtedly is to produce, with cholesterol, the peculiar semi-fluid, semi-solid state of protoplasm. Kingsbury (1912) describes mitochondria as intermediate products of metabolism, the structural expression of reducing bodies in respiration.

There seems, however, to be some evidence in the cricket egg that the mitochondria play here a rather specialized role in secretion. The fuchsinophile granules of the yolk, whether derived from the mitochondria of the follicle cells, as suggested, or not, are very similar to them in form and staining reactions; and are indistinguishable from any mitochondria which might be native to the egg. These are very intimately associated with the two larger kinds of globules in the oöcyte. Very similar granules also are found in the position of a cortex around the distal droplets. Although the resemblance, as regards staining, between the mitochondria of the follicle cell and the granules in these two locations is not exact, it is nevertheless so close as to support the presumption that they have the common origin which their form and distribution suggests.

The work of Gatenby and his associates, on insect ovogenesis and other invertebrate problems, has led them to similar conclusions as to the rôle of mitochondria in secretion. In a 1920 paper, Gatenby and Woodger go so far as to state that in nearly all eggs where fat granules are present, examination has revealed that such "yolk" is derived either from Golgi elements or from mitochondria. It seems possible that some of the present divergence of opinion as to the function of mitochondria in the cell is due to the diversity of the material used in the investigation of the problem. The absence in the cricket follicle cells of inclusions which will satisfy the assembled criteria of Golgi bodies, is another case in point.

VI. SUMMARY.

1. Follicle cells of the *B* stage rarely if ever divide during the development of the oöcyte up to and through the *A* stage, although these cells change in size and shape during that period.

2. The amitotic configuration of the nucleus may be regarded as an adaptation to the secretory activity of the cell, having as its main function the increase of nuclear and nucleolar surface.

3. Droplets of a fatty nature are elaborated by the follicle cells and passed in large numbers into the hæmocoel of the insect.

4. The mitochondria of the follicle cells represent an intermediate stage between lipoid granules appearing in the nucleus of the follicle cell and lipoid granules surrounding the yolk globules of the egg.

5. No inclusions corresponding entirely to current descriptions of Golgi bodies have been observed in egg or follicle cell.

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PLATE I.

Explanation of Figures.

1. Follicle-cells and a part of the *C*-egg, fixed by the Regaud method, and stained with acid-fuchsin and methyl green. Longitudinal section 3 micra in thickness; cells 1.67 micra in length. Drawn with camera lucida; enlarged to four times the area. Fuchsinophile granules in cytoplasm of follicle-cells and in cytosome of egg are figured black; globules staining with methyl green are stippled; dissolved globules are left white.

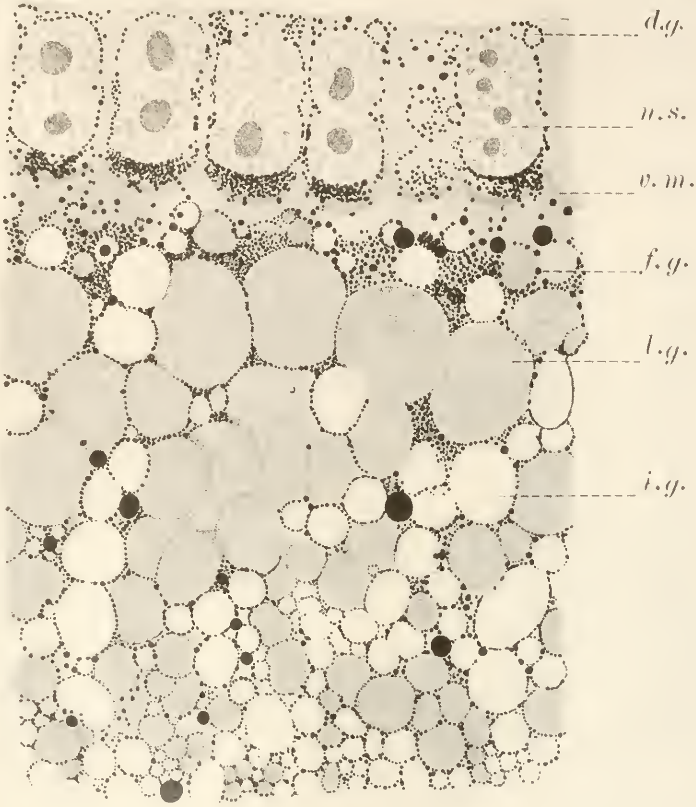
2. Follicle-cells and a part of the cytosome of the *B*-egg, fixed in Bouin's fluid and stained with Delafield's acid hæmotoxylin. Transverse section 5 micra in thickness; cells 3 micra in length. Drawn with camera lucida. Refractive granules of the proximal region of the cytoplasm are stippled more lightly than the large type of globules in the yolk substance.

3. Follicle-cells and a part of the yolk-substance of the *B*-egg, fixed in Champy's mixture of osmic and bichromate and pyrogallic acid; unstained. Longitudinal section 4 micra in thickness; cells 2.5 micra in length. Drawn with camera lucida. Two kinds of granules are shown in the nucleolus; refractive granules are shown in the proximal region of the cytoplasm of the follicle-cells.

Symbols.

d.g., distal droplet. *f.g.*, fuchsinophile granules. *i.g.*, intermediate globule. *l.g.*, large type of globule. *n.s.*, nuclear strand. *v.m.*, vitelline membrane.

Fig. 1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n.s.
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Fig. 2

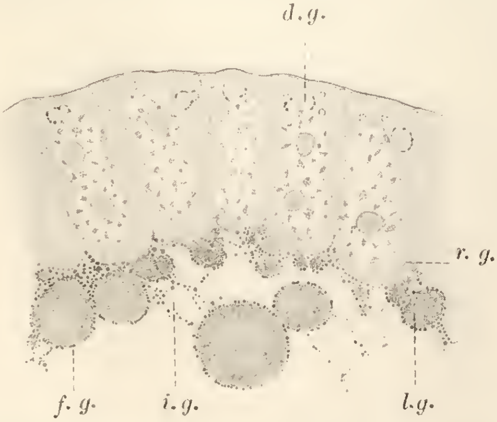
d.g.
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Fig. 3

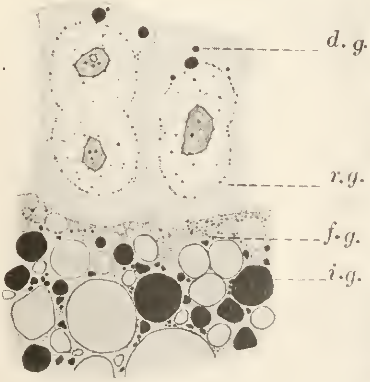


PLATE II.

Explanation of Figures.

4. Follicle-cells and a part of the cytosome of the *C*-egg, fixed by Da Fano's modification of Cajal's method for Golgi apparatus, and stained with Nile blue sulfate. Longitudinal section 4 micra in thickness; cells 1.5 micra in length. Drawn with camera lucida.

5. Follicle-cells of the *B*-egg, fixed by Kopsch's osmic acid impregnation method; unstained. Longitudinal section 3 micra in thickness; cells 3.5 micra in length. Drawn with camera lucida. Lighter granules in the cytoplasm are of the same character as the black ones, but lie at a lower level.

6. Follicle-cells of the *B*-egg, fixed by Champy's method, and stained with Delafield's hæmotoxylin. Longitudinal section 3 micra in thickness; cells 3.5 micra in length. Drawn with camera lucida. The nucleus tends to lie in the proximal region of the cell, while the distal region is occupied by a large number of granules.

Symbols.

d.g., distal droplet. *f.g.*, fuchsinophile granules. *i.g.*, intermediate globules. *l.g.*, large type of globule. *n.s.*, nuclear strand.

Fig. 4

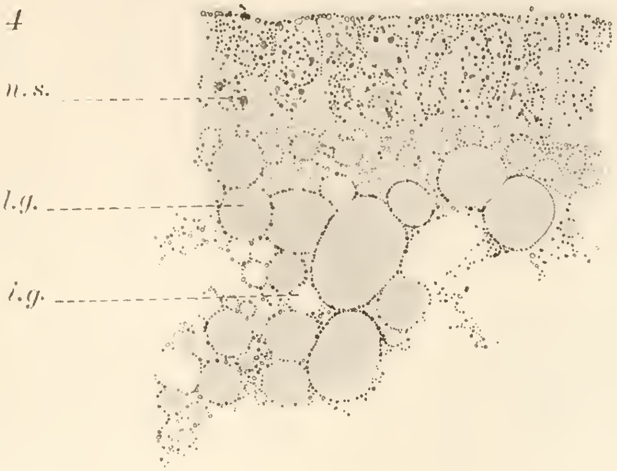


Fig. 5

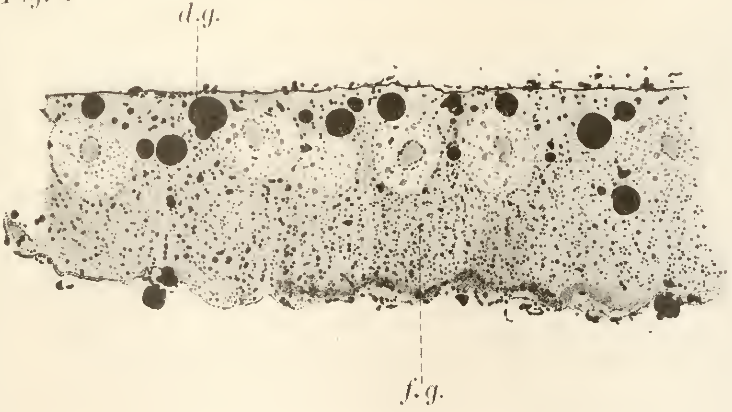


Fig. 6

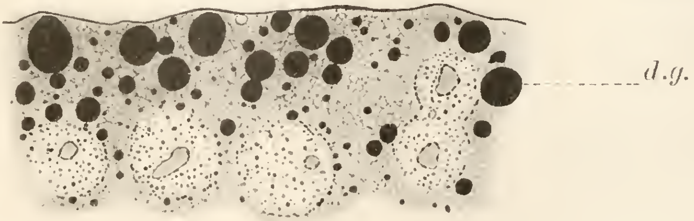


PLATE III.

(All drawings made approximately to scale.)

7. Diagrammatic sketch of a follicle-cell of the *B*-stage, unstained. The nucleus lies in the proximal region of the cell, and a large number of refractive granules occupy the distal region.

8. Camera lucida sketch of a cell from the *B*-follicle, unstained. The nucleus is in transition between the position in which its long axis is *perpendicular* to the surface of the follicle and the position in which it is *parallel* to the surface.

9. Diagrammatic sketch of a cell in the *C*-follicle, stained with janus green. Green-staining granules are shown outlining the nucleus and lying in the proximal region of the cytoplasm.

10. Surface view of a similar cell under the same conditions.

11. Camera lucida sketch of surface view of cells from the *A*-follicle, stained with neutral red and brilliant cresyl blue. The distal granules take the red stain.

12. Camera lucida sketch of surface view of cells from the *B*-follicle, stained with neutral red. The distal granules are red.

13. Camera lucida sketch of a cell from the *A*-follicle, unstained. The nucleus occupies the proximal region of the cell, and a number of highly refractive granules occupy the distal region.

14. Camera lucida sketch of surface view of a cell from the *A*-follicle, stained with methyl green. The distal granules are not stained. Some of them show small lobular irregularities, as if they were being formed by the coalescence of several smaller granules.

15. Camera lucida sketch of a whole egg-string. The black outline surrounding each egg represents the follicle. Beginning with the ripest in the series, the eggs are lettered in the order of their maturity. The *B*-follicle is the thickest of the series.

Fig. 7

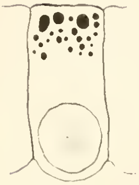


Fig. 8

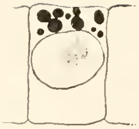


Fig. 9

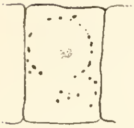


Fig. 10

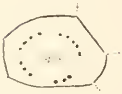


Fig. 11

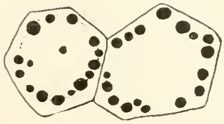


Fig. 12

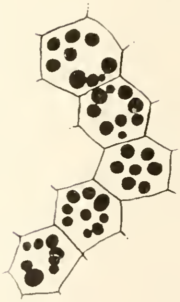


Fig. 13

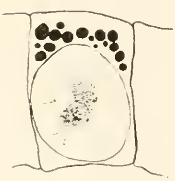


Fig. 14

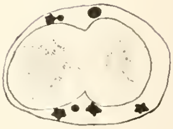
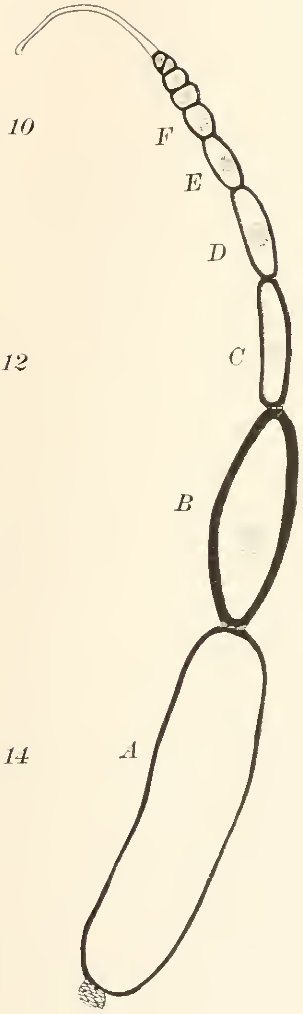


Fig. 15



MARGARET MURRAY.

VITAL STAINING AND REDUCTION OF VITAL STAINS BY PROTOZOA.

ELERY R. BECKER,

IOWA STATE COLLEGE.

It is well known that fresh animal and plant tissues and milk have the power of reducing methylene blue to the leuco-base. This is supposed to be brought about by the hydrogen liberated in the decomposition of water by a reducase. The reducase in turn requires the presence of Schardinger's enzyme, an oxidizable substance, and water. (See Bayliss, p. 588.)

It is not generally known that protozoa, too, may possess the reducase. The demonstration of its presence requires that they be kept in a weak solution of the dye in an oxygen free medium. The method used by the writer was a hanging drop of ciliates and dye solution in an Engelmann chamber, through which a stream of hydrogen was passed. The drop was observed through the compound microscope. The ciliates used were *Opalina sp.?* from the bullfrog tadpole and *Paramecium caudatum*, representing both parasitic and free-living modes of life. Fifteen dyes were tested out on *Opalina*, and one on *Paramecium*. The best reductions are obtained if the hydrogen gas is first passed through heated platinized asbestos to remove the oxygen.

Janus Green.—*Opalina* in suspension of tadpole feces from posterior intestine of tadpole were suspended in a drop of Janus green (1 to 15,000) made up in 0.3 salt solution. Apparently the dye did not stain any substance in the ciliate. After displacing the air in the chamber with hydrogen, a number of the *Opalina* contain pink granules, due to reduction of the Janus green (diethylsafraninazodimethylanilin) to diethyl safranin. On exposure to air the pink substance did not change its color. Further reduction to the leuco-base could not be effected. Not over one fourth of the *Opalina* at any one time could reduce the dye. The reduction was also accomplished in a muscle chamber.

Brilliant Cresyl Blue.—Stains *Opalina* light blue in dilutions of 1 to 50,000. In the reduction to the leuco-base, there is an intermediate greenish hue. All the *Opalina* took the stain and showed reduction of the dye. On readmittance of air, the blue reappeared.

A number of *Nyctotherus* stained blue with this dye, but showed no reduction phenomena.

Nile Blue.—Stains *Opalina* blue-green in dilutions of 1 to 30,000. On reduction to the leuco-base the color disappears. Readmittance of air causes green stains to reappear.

Toluidine Blue.—Stains *Opalina* violet. Reduced to leuco-base. Color reappears on readmittance of air. Not all organisms took the stain.

Bismarck Brown.—Does not stain *Opalina.*

Methyl Green (Vital).—Living *Opalina* do not stain, even in dilutions of 1 to 1,000. In dilutions of 1 to 300 the dead ones stained a beautiful violet, but showed no reduction of the dye.

Vital Red.—1 to 2,000 does not stain *Opalina.*

Isamine Blue.—Does not stain *Opalina* in dilutions of 1 to 15,000 and 1 to 20,000.

Trypan Blue.—Does not stain *Opalina* in dilutions of 1 to 10,000 and 1 to 20,000.

Trypan Red.—Same results as *Trypan blue.*

Carminate of Sodium.—Same as *Trypan blue.*

Fuchsin (Neutral).—Same as *Trypan blue.*

Rhodamine.—In dilutions of 1 to 20,000 this dye will stain *Opalina* a light pink. No reduction of this dye could be accomplished, although in a test tube the dye could be reduced to the leuco-base by means of zinc and HCl.

Neutral Red.—Stains *Opalina* and certain granules and food vacuoles in *Paramecium* pink, but no reduction could be accomplished in the hydrogen chamber.

Methylene Blue.—This dye stains *Opalina* blue almost at once. It takes about ten minutes in order for it to stain *Paramecium* well. A comparison of the rate of reduction of the dye by the two ciliates would have been interesting, but the problem was complicated by the fact that *Opalina* completely and uniformly reduced the dye with which it is stained to the leuco-base within three or four minutes, while *Paramecium* does not show so uniform a reduction. The dye is retained for a considerable length of time about the food vacuoles and granular substances in the protoplasm. This makes exact comparison of the rates of reduction difficult. But it is evident that the general protoplasm of *Paramecium* has become quite colorless at about the time the *Opalina* has reduced the methylene blue to the leuco-base. This fact makes it improbable that the *Opalina* has any advantage over *Paramecium* in its parasitic mode of life in the way of obtaining oxygen for respiration from the decomposition of water.

In addition to these experiments with *Opalina* and *Paramecium*, Janus green of about 1 to 15,000 dilution was tested out on the protozoa of the intestine of the termite *Reticulitermes flavipes*. These protozoa ingest the particles of wood which the termite eats. Cleveland (1924) has supplied the final proof that these protozoa are instrumental in assisting the termite to digest its cellulose diet. After a short time the Janus green stains the small particles of wood in the bodies of the various protozoa a light green. If the coverslip is sealed tightly with vaseline, the color changes to pink, and finally to the leuco-base. This suggests the possibility that oxidation of some of the products of digestion of the wood takes place in the vicinity of the food vacuoles, or that oxygen is used in some way in the digestion of the wood itself.

CONCLUSION.

Opalina has the power of reducing the vital dyes Janus green, brilliant cresyl blue, Nile blue, toluidene blue, and methylene blue. *Paramecium* shows similar reduction properties. This shows the presence of a reducase in the protoplasm of protozoa. The wood-ingesting protozoa of the termite's intestine possess a reducase in the food vacuoles, as proved by the reduction of Janus green in these parts of the cell.

The possession of this reducase is not an adaptation to the parasitic mode of life in the intestine where the oxygen pressure is low for free living protozoa may also possess it. The results are too meagre to state definitely whether the parasitic protozoa may have the advantage over free living protozoa in possessing greater amounts of it.

My thanks are due to Professor E. N. Harvey, of Princeton University, for his suggestions in regard to the apparatus.

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THE INTERRELATIONS OF PROTOZOA AND THE UTRICLES OF UTRICULARIA.[1]

R. W. HEGNER.

References to the presence of "Infusoria" within the utricles of Utricularia occur in several publications on insectivorous plants, but no one has hitherto studied the interrelations of these protozoa and the plants. The situation suggests various questions, such as (1) are the protozoa intruders that act as scavengers feeding on the dead bodies of other captives; (2) are they captured by the plants and used as food; (3) are they adventitious residents that slowly starve to death within their prison-like walls; or (4) are they "normal" inhabitants, possibly differing in species from free-living protozoa, that maintain more or less intimate parasitic or symbiotic relations with the utricles. If the last named possibility is correct these protozoa may be thought of as living within a sort of primitive plant stomach the liquid contents of which consist of digestive ferments and a certain quantity of organic materials in various stages of diges-

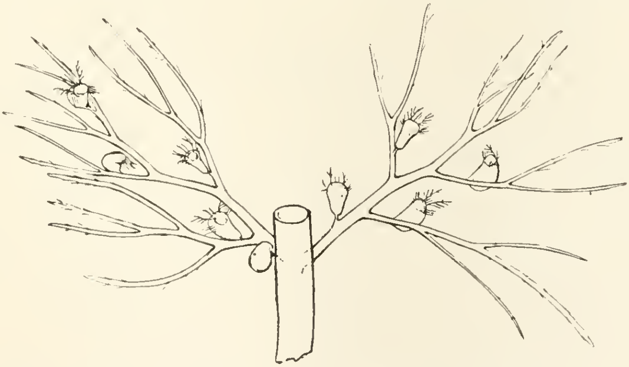

FIG. 1. Part of a branch of Utricularia showing bladders. × 4.

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[1] From the Department of Medical Zoölogy, School of Hygiene and Public Health, Johns Hopkins University, and the Mt. Desert Island Biological Station, Salisbury Cove, Maine. The writer is indebted to Mrs. Ethel Norris Rask for making the drawings.

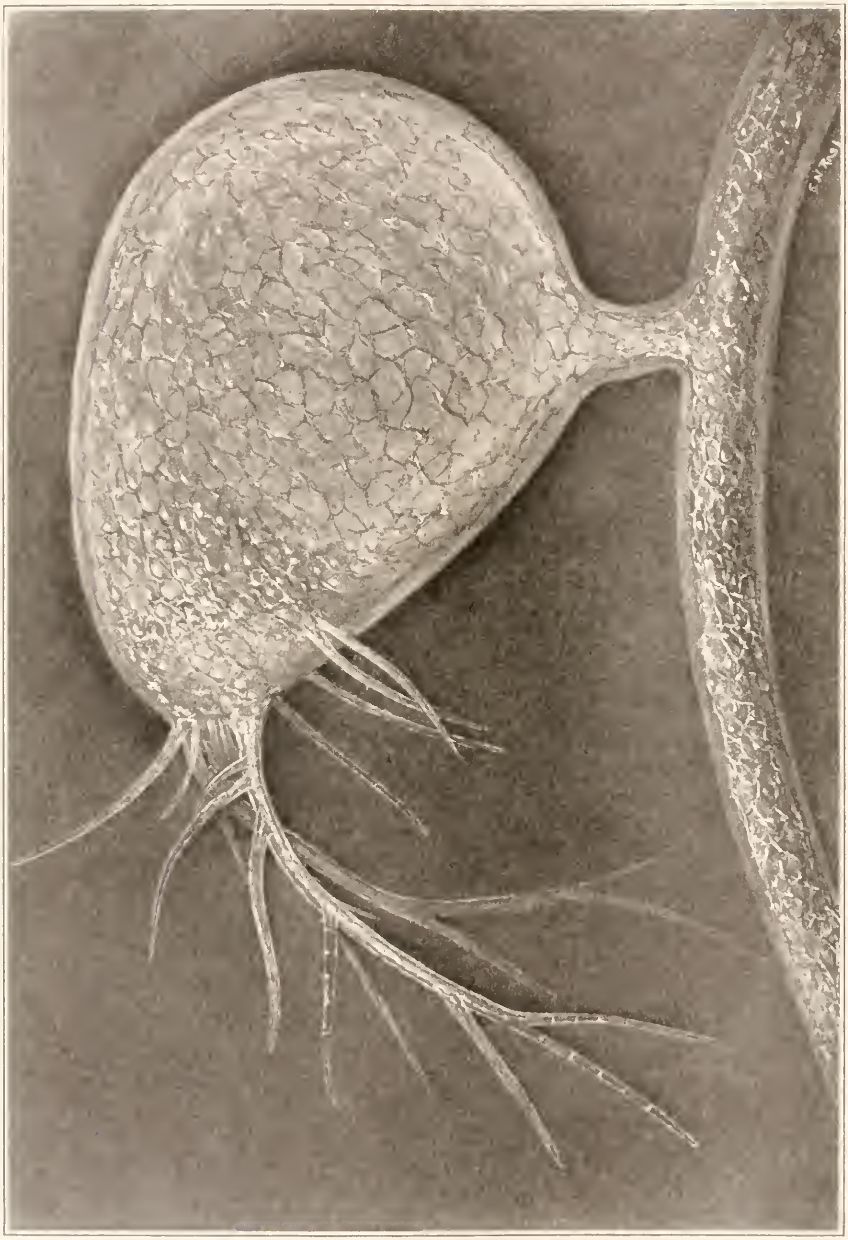

FIG. 2. Side view of a normal bladder with side walls compressed. × 70.

tion. The reactions of intestinal flagellates to their environment within the digestive tract of their hosts represent a very interesting phase of the subject of host-parasite relationships which has occupied the attention of the writer for the past five years. The studies described below were undertaken, therefore, not only because of the intrinsic interest of the subject, but also because they might throw some light on the factors involved in the relations of intestinal flagellates to their entozoic environment.

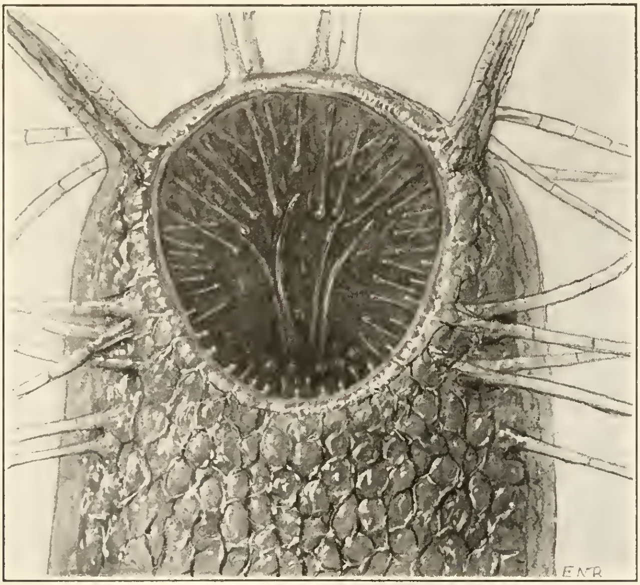

FIG. 3. View looking down into the vestibule of a bladder showing the two pairs of bristles and various glandular hairs on the valve, and other glandular hairs extending out from the sides of the vestibule. × 110.

Specimens of *Utricularia vulgaris* v. *americana* Gray live in fresh-water ponds and are often present in great abundance floating near the surface. A typical branch is shown in Fig. 1. The utricles or bladders are borne at intervals along the branches. Fig. 2 represents a side view of a bladder showing the bristles which extent out above and on either side of the mouth opening. Within the mouth is a shallow vestibule at the bottom of which is a thin, flexible valve which is attached at the top and sides but free below, where it rests tightly against an inward projecting mass of cells. This valve can open only inward. A view looking down upon the mouth opening is shown in Fig. 3. This view reveals two pairs of bristles projecting from the valve near the lower edge, a row of glandular hairs near by, and numerous glandular hairs attached to the valve near its upper edge. Extending out into the vestibule from the side walls are many glandular hairs of various lengths. The bladders vary in size from about 1 mm. to 4 mm. in length. They are usually filled with liquid but may contain bubbles of gas. They were once thought to be floats which kept the plant near the surface of the water, but the presence of small animals within them soon placed them among the "insectivorous" plants. A typical bladder when "set" and ready to function as a trap for the capture of minute aquatic animals is laterally compressed as shown in Fig. 4. Just after the capture of an organism the bladder is much distended as indicated in Fig. 5. Darwin (1875) noted that the bladders varied much in thickness according to the quantity of water contained in them and that they "are always somewhat compressed," but as will be explained later he did not realize the significance of the compressed and expanded conditions.

The remarkable ability of these bladders to capture free-swimming aquatic organisms is well indicated by an examination of a typical branch. The branch studied consisted of a main stem 110 cm. long bearing 4 side branches the combined length of which was 110 cm. The number of bladders per cm. on the main stem was estimated as 90, and on the side branches, as 36. The total approximate number of bladders was therefore 13,860. The organisms within 10 mature bladders selected at random were counted. These consisted of entomostraca, insect larvæ, rotifers, usually euglenas and often other protozoa. Entomostraca represented the principal article of food; these ranged from 6 to 22 per bladder with an average of 12. A conservative estimate of the number per bladder would be 10, which would place the total number of entomostraca captured by this part of the plant at about 150,000. The bladders live for some time and as the victims die and break down fresh specimens are captured, so that the total number captured by the plant during

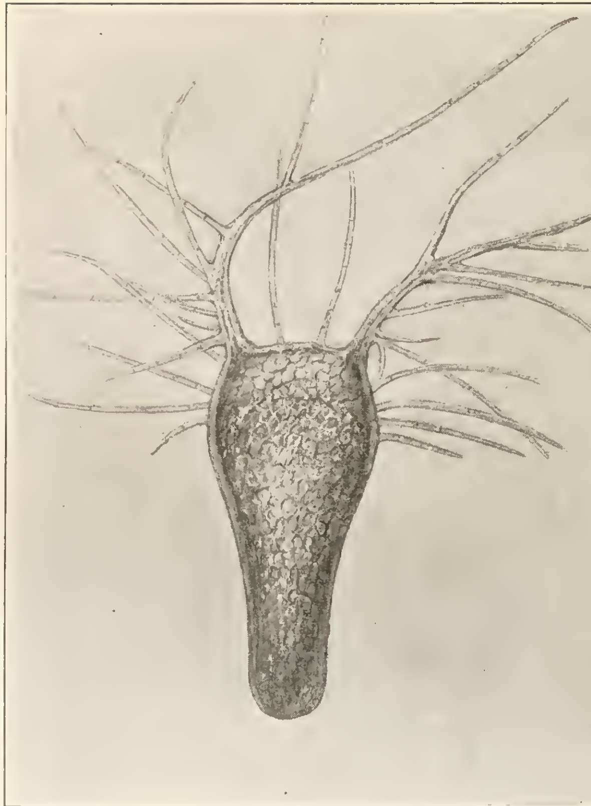

FIG. 4. Bladder compressed and ready to capture any animal that provides the proper stimulus. × 70.

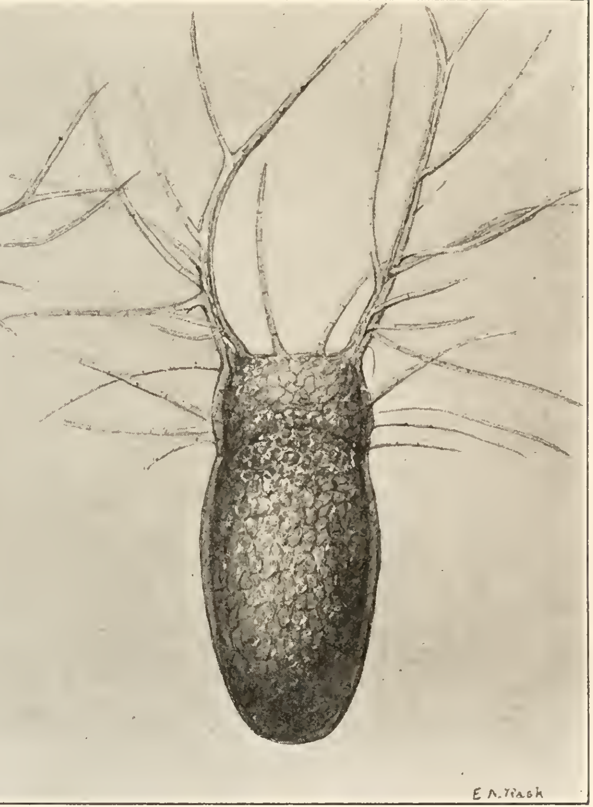

FIG. 5. Bladder expanded after the capture of an organism. × 70.

the summer must be enormous and must add very materially to its nutriment if the organic material is absorbed as Darwin seems to have proved.

The protozoa that are able to live within the bladders are few both in number of species and number of individuals. Euglenas, as noted elsewhere, are rather common inhabitants. Minute ciliates and flagellates as well as small amœbæ may also be present. Darwin mentions that, "In all cases the bladders with decayed remains swarmed with living Algæ of many kinds, Infusoria, and other low organisms, which evidently lived as intruders." He does not seem to have realized that some of these Infusoria might have been captives instead of intruders.

So far as I have been able to discover the correct method of capture of organisms by the utricularia bladder has never been described.* Only after 69 of a total of 91 experiments were carried out did I discover the significance of the compressed and distended condition of the bladders, and this was finally revealed to me because of the large size of the bladders borne by the plants found near Bar Harbor, Maine. Paramecia were found to be easily and quickly captured, but many hours were spent in attempts to determine exactly how this was accomplished. The bristles around the entrance seemed rather to hinder than to guide them to the opening, since many specimens that might have entered gave the avoiding reaction and swam away when they encountered these bristles. Perhaps as Darwin suggests "their use probably is to prevent too large animals from trying to force an entrance into the bladder, thus rupturing the orifice." Only a very few of the paramecia that swam into the entrance were captured and those that were captured were taken in so rapidly that it was impossible to determine whether they forced their way in or the valve opened to receive them. Darwin says, "Animals enter the bladders by bending inwards the posterior free edge of the valve, which being highly elastic shuts again instantly." This describes only in part the method of capture. The solution of the problem was reached when a compressed bladder whose valve was touched with a needle at the lower, free edge suddenly became distended by an inrush of water. A similar reaction occurred in every compressed bladder similarly treated, but no change was evident when the valve of a distended bladder was touched with a needle. Several experiments were carried out to test the capacity of bladders to recover their compressed condition after being distended by the touch of a needle.

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* Since this paper was written I have had better access to literature and find that the method by which Utricularia captures its prey was more or less correctly described independently by Brocker in 1911 (Ann. de Biol. Lacustre, VI), by Ekambaram in 1916 (Agric. Jour. India) and by Withycombe in 1924 (Jour. Linn. Soc. Bot., 46). I am indebted to Mr. A. F. Skutch for the reference to Brocker's paper.

Experiment M. 15.—Five branches were selected and a number of bladders on each were distended by touching the lower part of the valve with a needle: branch *a*, 7 bladders; *b*, 4; *c*, 4; *d*, 4; *e*, 4. Forty minutes later these bladders had all regained a compressed condition. Several of them contained large insect larvae and many of them crustaceans in various stages of decay, but apparently the previous capture of food had no influence on their assumption of the compressed state. These bladders were all again distended as before. When reëxamined in 20 minutes all bladders on branches *a*, *b* and *c* were found to be compressed and on each of branches *c* and *d*, 2 were completely and two partially compressed. They were all again distended. Ten minutes later none of the bladders had entirely recovered. Fifteen minutes later some had entirely recovered, some almost and others only slightly. At this time the bladders were all again distended. Two hours and 15 minutes later all bladders on branches *a*, *b*, and *c* had recovered and became distended when touched with a needle, but only 2 on each of branches *c* and *d* were compressed. Two hours and 45 minutes later all bladders on branches *a*, *b*, and *c* had recovered except 2 on each which had only partly recovered; none recovered on branch *c*; and 2 recovered on branch *d*. All bladders that were entirely or partly compressed were distended with a needle at this time. Seventeen hours later or 24 hours after the beginning of the experiment, on branch *a*, 4 bladders had entirely recovered and 3 had partly recovered but became fully distended when touched with a needle; on branch *b*, 2 had entirely recovered and 2 had partly recovered, all became distended when stimulated; on branch *c*, 2 had entirely recovered, one had partly recovered and one was not compressed at all; on branch *d*, none of the experimental bladders had recovered, and it was noted that all the other bladders on this branch were distended, due possibly to some change in the physiological condition of the branch; on branch *e*, one partly recovered, the other three were fully distended.

This experiment gives some idea of the rapidity with which the bladders may capture their prey. The length of time from the capture of an organism until the trap is set for another is apparently about 20 minutes, and it is evident that the capture of organisms may occur a number of times in one day. The failure of some of the bladders to regain the compressed condition after being distended several times may have been due in part to injury from the needle and in part to the separation of the branches from the rest of the plant. The latter seems probable at least in the case of branch *d*.

Experiment M. 22.—This experiment was carried out with 10 detached bladders, 5 from each of 2 stems. Bladders may be handled quite roughly without their becoming distended and all of these 10 were successfully detached in the compressed condition. They were then distended with a needle. Forty minutes later 8 of them had again become compressed and the remaining 2 had partially regained their compressed condition.

These results show that the processes of both distention and compression occur in detached bladders.

Compressed bladders may become distended in several ways besides that of touching the valve with a needle. When pressure is applied to either side of the walls of the entrance by squeezing the anterior end of the bladder between the arms of a fine forceps, thus opening the valve, the bladder becomes distended. Distension similarly occurs when pressure is applied antero-posteriorly. Compressed bladders may also be made to distend by puncturing one side with a needle thus allowing the entrance of liquid from outside.

The difference in the amount of liquid in compressed and distended bladders is considerable. Five branches were selected and 3 large compressed and 3 large distended bladders were detached from each, dried on the outside with filter paper and the contents withdrawn with a fine pipette. It was found that the contents of the 15 distended bladders amounted to approximately 88 per cent. more than that of the 15 compressed bladders. That is, when a bladder becomes distended it draws in a volume of the outside medium equal to about 88 per cent. of that already present. The process of distention is remarkably rapid and therefore the outside medium enters with considerable force.

The amount of suction caused by the expansion of the walls of the bladder is indicated by the following experiments. Paramecia were killed with heat and placed at various distances from the entrance; the valve was then touched with a needle. It was found that specimens as far away as 2 mm. were easily drawn into the bladder. A dead insect larva 2 mm. long was dissected out of one bladder and gently pushed against the valve of another bladder; the entire larva was instantly drawn in, although the bladder was hardly longer than the larva. A living insect larva of the same length was placed at the entrance of another bladder; when it touched the lower edge of the valve it was also entirely and instantly drawn in. Part of a slender branch of a utricularia plant was presented to another bladder; this was drawn in until one end touched the posterior end of the bladder and the other end projected from the entrance. No more of this branch was taken in during the succeeding 24 hours.

It is evident from these experiments that the animals taken into the bladders do not try to force an entrance, but are sucked in when, by their movements, they stimulate the plant in such a way as to cause the valve to open and the walls of the bladder to expand. The exact mechanism of this process is still to be worked out.

Other species of Utricularia probably capture their prey in a similar manner. One of these *Utricularia intermedia* Hayne, which bears bladders on separate leafless branches that lie on the bottom was examined at Salisbury Cove, Maine; its bladders were compressed, but expanded when stimulated with a needle just as did those of *U. vulgaris* v. *americana*.

Various investigators have attempted to determine how utricularia bladders capture their prey. Darwin carried on experiments somewhat similar to those described above but failed. That he very nearly succeeded is evident from the following account of his experiments.

> "As I felt much difficulty in understanding how such minute and weak animals, as are often captured, could force their way into the bladders, I tried many experiments to ascertain how this was effected. The free margin of the valve bends so easily that no resistance is felt when a needle or thin bristle is inserted. A thin human hair, fixed to a handle, and cut off so as to project barely ¼ of an inch, entered with some difficulty; a longer piece yielded instead of entering. On three occasions minute particles of blue glass (so as to be easily distinguished) were placed on valves whilst under water; and on trying gently to move them with a needle, they disappeared so suddenly that, not seeing what had happened, I thought that I had flirted them off; but on examining the bladders, they were found safely enclosed. The same thing occurred to my son, who placed little cubes of green box-wood (about 1/60 of an inch, .423 mm.) on some valves; and thrice in the act of placing them on, or whilst gently moving them to another spot, the valve suddenly opened and they were engulfed. To ascertain whether the valves were endowed with irritability, the surfaces of several were scratched with a needle or

brushed with a fine camel-hair brush, so as to imitate the crawling movement of small crustaceans, but the valve did not open. We may, therefore, conclude that animals enter merely by forcing their way through the slit-like orifice; their heads serving as a wedge."

Mrs. Treat (1875) seems to have been the first to study the method of capture employed by the utricularia bladders. Her description, in part, is as follows: "They (little animals) would sometimes dally about the open entrance (the vestibule) for a short time, but would sooner or later venture in, and easily open or push apart the closed entrance at the other extremity. As soon as the animal was fairly in, the forced entrance closed, making it a secure prisoner." "I never saw even the smallest animalcule escape after it was once fairly inside the bladder."

According to Büsgen (1888) animals do not force their way into the bladder between the edge of the valve and the wall of the bladder as supposed by Darwin. They were able to creep about on the outer surface of the valve for considerable periods without being engulfed but suddenly the valve would open and close again and the animals would disappear within. He believed that the mucus on the wall of the bladder where it meets the valve helps to precipitate the prey into the cavity of the bladder and that the mucus and bristles at the mouth of the bladder may stimulate the animal to violent movements which cause the valve to open. The sudden opening of the valve led him to think that he was dealing with a phenomenon of irritability, but he found as did Darwin that stimulation of the bristles with a needle or brush did not cause the opening of the valve. Büsgen almost arrived at the correct method employed by the bladders in capturing animals but apparently did not note the distension of the walls of the bladder and hence missed the essential feature of the process.

Goebel (1891) agreed with Mrs. Treat and with Darwin that the captured animals force their way into the bladders. Certain of the glandular hairs were supposed by him to be attractive to microörganisms, and other glandular hairs to be so located as to aid the animals to enter the bladder.

Mueller (1925) in an article entitled "The Rotifer Group" published two figures showing utricularia bladders. The legends of these figures read in part as follows: (1) "If a rotifer or other tiny pond animal chances to rest upon this door, it immediately slips through the slot which quickly closes upon the prisoner. The animal swims about inside the utricle and finally dies." (2) "Larva of harlequin fly (*Chironomus*) just captured by plant. This larva was feeding upon food particles adhering to the branched spines projecting from the vesicle, and gradually worked its way down the stem until it touched the slippery trapdoor which straightway opened and caught it by the head. Because of the downward-pointing hairs lining the vesicles the struggles of the larva merely draw it further into the trap." The method of capture described in these legends does not agree with that I have found to be true of the species I have studied. Apparently the legends were written without any actual observations of the process. Furthermore I know of no "downward pointing hairs lining the vesicles" that might draw an insect larva into the bladder.

The most recent papers on the capture of organisms by utricularia are by Brumpt (1925) and Langeron (1925). Brumpt notes the capture of mosquito larvæ by the bladders and stresses their importance in the control of malaria by mosquito reduction but does not discuss the method of capture. Langeron states that animals penetrate easily the bladders of both aquatic and terrestrial species of Utricularia and that only a slight pressure on the valve is sufficient to precipitate them into the cavity, but does not describe the process further.

Organisms once captured appear never to escape. Frequently, as Darwin noted, insect larvæ are sometimes too long to be drawn in at one time, and bladders are to be found with part of the body of the larva inside and the rest projecting from the entrance. Brumpt (1925) has shown that mosquito larvæ are often drawn in tail first and on account of their large size are usually to be found with their heads extending out from the entrance. Brumpt accounts for this by the habit of certain insect larvæ of inserting the end of the tail into crevices. In this way he supposed that insect larvæ forced their way into the bladders. A more simple explanation, now that the method of capture is known, is that the ventral brush of the anal segment which the mosquito larva moves briskly in swimming stimulates the bladder to action and that it is therefore the posterior end that is drawn in first.

As already noted, a distended bladder regains its compressed condition in about 20 minutes. How this is accomplished by the plant I do not know but it involves the absorption by or expulsion from the bladder of about 88 per cent. of its liquid contents. Several experiments were performed in an attempt to determine whether liquid passes into and out of the bladders at any time except when organisms are captured.

Experiment 66.—Seven branches bearing from 6 to 19 bladders each were immersed for $2\frac{1}{2}$ hours in aqueous solutions of carmine, india ink, eosin, safranin, and crystal violet. All bladders were deeply stained in safranin and crystal violet. The walls were not stained in the other solutions but many of the bladders contained the coloring matter; 4 of 9 bladders in carmine contained red granules; 8 of 14 in eosin were filled with this solution; and 10 of 15 took in India ink.

Experiment 67.—Branches bearing 100 bladders were immersed in an aqueous solution of eosin for 3 hours. The solution was found within 69 of these bladders at the end of this period, mostly in young and medium sized specimens.

Experiment 68.—Branches bearing 52 bladders were immersed in a paramecium culture for 2 hours and then in an eosin solution for $2\frac{1}{2}$ hours. Paramecia were captured by 12 bladders and became stained with eosin in 11 of them. Nine of the bladders that did not capture paramecia took in eosin and 32 did not.

Experiment 69.—Nine branches bearing 79 bladders were immersed in a paramecium culture 2 hours and then in an eosin solution 2 hours. Paramecia were captured by 67 bladders and became stained with eosin in 65 of them. The 12 bladders that did not capture paramecia all took in eosin.

Experiment M. 20.—Compressed bladders were placed in an eosin solution and expanded thus adding this solution to their contents. They were then washed in water, placed in hollow ground slides and examined. None of these bladders recovered the compressed condition.

These experiments prove that solutions of eosin, carmine and India ink are taken in by bladders that have not captured paramecia and eosin solution by bladders that have captured paramecia. That this is due to spontaneous expansion of the bladders is possible but not probable. How the solutions were taken in is still in doubt. This is true also regarding the passage of liquid out of the bladders. This is a problem for the plant physiologists to solve, and work on it is now in progress.

After discussing the probable function of the glands located around the exterior of the entrance Darwin concludes that these "are adapted to absorb matter from the putrid water, which will occasionally escape from bladders including decayed animals." He remarks, however, that, "The valve fits so closely, judging from the result of immersing uninjured bladders in various solutions, that it is doubtful whether any putrid fluid habitually passes outward. But we must remember that a bladder generally captures several animals, and that each time a fresh animal enters, a puff of foul water must pass out and bathe the glands." On the contrary, my experiments prove that when an animal enters a bladder, medium is taken in rather than forced out, and that fluid passes outwards between the time of one capture and that of the next succeeding capture, *i.e.*, during the period of compression. Darwin's main conclusion may, therefore, be correct, provided the glands around the entrance absorb material if this is forced out of the valve during the compression of the bladder.

Experiments with Euglenas.

Euglenas are of frequent occurrence in the bladders of Utricularia plants in nature. In 1922 euglenas were found to be present in almost all of the bladders of Utricularia plants obtained from a pond on the campus of the Johns Hopkins University and almost every bladder was infected on plants that had been kept in a dish in the laboratory for several months. The number present in a single bladder as determined by counting those in ten bladders selected at random, ranged from 8 to 510, with an average per bladder of 215 (Hegner, 1923). The pond from which these plants were taken contains large numbers of euglenas at certain times of the year and these bladders may have become infected with these free-living specimens. It was believed, however, at that time, that the utricularia bladders contained digestive ferments and on this account might successfully withstand the conditions within the digestive tract of tadpoles in which euglenas had been proved to be normal inhabitants (Hegner, 1923, Wenrich, 1923). When fed to tadpoles, however, these euglenas were promptly digested. Euglenas were also noted in many of the bladders on plants of *Utricularia vulgaris* var. *americana* collected near Bar Harbor, Me., although not in as great numbers as in those on plants collected in Baltimore.

Utricularia bladders lose their infection with euglenas when kept for a long period in the laboratory. Bladders of Utricularia were used for experimental purposes in the spring of 1925 that were taken from plants that had been kept in large stender dishes in the laboratory for about eight months. These bladders were almost entirely free from euglenas although when first brought into the laboratory the bladders were infected with large numbers. This may be accounted for as follows. Bladders do not live very long, but gradually loose their chlorophyll, break away from the plant and drop to the bottom. Here they disintegrate in course of time thus liberating any euglenas contained in them. These euglenas either live in the water or are captured by the new bladders that are continually developing. Their habitat within the bladders is very restricted and their escape from the old bladders and reinfection of new bladders difficult. Probably for these reasons, the number of euglenas decreased in plants that were kept under laboratory conditions.

Free-living euglenas are captured by bladders and live normally and multiply within bladders. Bladders were immersed in cultures containing many free-living euglenas or euglenas were injected into them with a fine pipette.

Experiment 2.—One of ten detached bladders became infected in 24 hours with one active free-living euglena.

Experiment 4.—Thirteen attached bladders failed to become infected in 48 hours.

Experiment 23.—Eleven of twenty detached bladders became infected in 40 hours. The numbers present in these bladders were as follows: 3 bladders with one each; 3 with 2 each; one with 3; and 4 with 6 each. The average number per bladder was 3.3. These bladders were examined at intervals of 3 days for 9 days. They all remained infected during this period. At the end of 3 days the number had increased in 6 of the bladders; the average per bladder increased from 3.3 to 6.7. A further increase took place during the next three days, the average rising from 6.7 to 15.1. During the succeeding three days this average increased from 15.1 to 20.1 per bladder. The numbers present in the individual bladders at this time were 50, 40, 30, 30, 16, 10, 8, 7, 6 and 4. One infected bladder had broken down and allowed the euglenas to escape.

Experiment 20.—Euglenas were injected with a fine pipette into 10 bladders attached to 4 stems, each bladder receiving from one to eight specimens. These were examined on 7 days during the succeeding period of 9 days. The number increased in 7 of the ten bladders; the other 3 bladders became detached before the observations were completed.

These experiments show that bladders may become infected with free-living euglenas and that these not only are not destroyed within the bladders but appear to move about normally and reproduce. Normal activity and reproduction also occur in attached bladders into which euglenas have been injected. These results suggest that the euglenas found in bladders in nature are free-living species that were captured by the bladders and were unable to escape and that these euglenas are either able to withstand any digestive ferments contained in the bladders or do not stimulate the production of such ferments by the bladder cells.

Euglenas are not injured in bladders that have captured and killed paramecia.

Experiment 30.—Thirty-nine bladders attached to 4 stems were immersed in a euglena culture for three hours and then in a paramecium culture for one hour. Seventeen of the bladders became infected with paramecia, from one to 17 in each bladder, the average per bladder being 3.6. Four of the bladders became infected with one paramecia each and three of these also contained euglenas, one with 5 and 2 with 2 each. In every case the paramecia had been killed but the euglenas were active.

Experiment 33.—Seventeen bladders attached to 2 stems were immersed in a paramecium culture for $2\frac{1}{4}$ hours; 13 became infected with paramecium as follows: 5 with one each, 5 with 2, one with 4, one with 6, and one with 8. After an interval of 4 hours when all the paramecia had been killed and many had already disintegrated euglenas were injected with a capillary pipette into each of the infected bladders. Nineteen hours later the euglenas within these bladders were active and apparently normal.

Experiment 51.—Forty-four bladders attached to 6 stems were immersed in a mixed culture of paramecia and euglenas and allowed to remain for 3 hours. Twenty-one bladders became infected with paramecia as follows: 18 with one each, 2 with 2, and one with 3; twenty-nine bladders became infected with euglenas; and 14 became infected with both. These bladders were examined daily during the four succeeding days and active euglenas were observed in 13 of the 14 bladders that captured paramecia. In the 14th bladder the euglenas were not moving. The euglenas were active in 14 of the bladders that had not captured paramecia and quiet in the 15th. Seven bladders captured paramecia but no euglenas and 6 bladders did not become infected with either.

Experiment 50.—In this experiment, which was initiated for another purpose, one bladder was found to contain active euglenas in the presence of disintegrating paramecia.

In the above experiments paramecia and euglenas became associated within the bladders of Utricularia in three different ways: (1) bladders were first infected with euglenas and then with paramecia, (2) bladders were first infected with paramecia and then euglenas were injected into them, and (3) bladders became infected with both in a mixed culture of paramecia and euglenas. The results show that regardless of the method of infection the euglenas remain active in bladders that have brought about the death and disintegration of paramecia, and that any digestive ferments that might have been produced by the bladder stimulated by the presence of paramecia, are not injurious to the euglenas.

Liquid from bladders which have captured and killed paramecia does not kill euglenas.—In Experiment 36, the liquid was squeezed out of 25 bladders that had captured and killed paramecia. Euglenas added to this liquid were still active 18 hours later.

Experiments with Paramecia.

The bladders of utricularia capture and kill paramecia. A preliminary experiment proved that paramecia are readily captured and killed by utricularia bladders.

Experiment 1.—A branch bearing 14 bladders was immersed in a culture containing large numbers of paramecia. When examined on the following day, 22 hours later, all bladders were found to contain paramecia only one of which was alive. It was evident that a number of specimens had disintegrated so the exact number captured could not be determined, but there were at least 4 bladders with one specimen each, 3 with 2, 5 with 3, and 2 with 5. The specimen that was still alive died within one hour and was beginning to disintegrate 2 hours later. On the next day, 48 hours after the experiment was begun, the only traces of paramecia remaining in the bladders, which had in the meantime been taken out of the paramecium culture, were fine granular masses representing thoroughly disintegrated paramecia. It is evident from this experiment that protozoa as small as paramecium are captured by the bladders and are killed and disintegrate within a few hours.

Paramecia are captured by either bladders that are attached to or detached from the utricularia plant in as short a time as 8 minutes. Branches bearing bladders were placed in paramecium cultures or bladders were detached from the plant and immersed. They were taken out; washed in water, and examined at various intervals. The following data were obtained from 8 experiments.

Total number of bladders	180
Total number positive for paramecia	56 or 30%
Total number negative for paramecia	124 or 70%
Number of bladders attached to plant	150
Number of attached bladders positive for paramecia	47 or 30%
Number of detached bladders	30
Number of detached bladders positive for paramecia	9 or 30%

Time required to capture paramecia, from 8 minutes to 2 hours; average 56 minutes.

Time required for attached bladders to capture paramecium, from 30 minutes to 1½ hours; average, 56 minutes.

Time required for detached bladders to capture paramecia, from 8 minutes to 2 hours; average, 59 minutes.

Number captured per positive attached bladders, from one to 6; average, 1.54.

Number captured per positive detached bladder, from one to 4; average, 1.56.

These data indicate that no difference exists between attached and detached bladders so far as the capture of paramecia is concerned. Regarding the length of time required for the capture of paramecia the data are not accurate since the bladders could not be kept under continuous observation; but in a culture containing active paramecia about 30 per cent. of the bladders succeeded in capturing one or more paramecia within one hour.

The number of paramecia captured depends on the character of the culture medium. Six experiments were carried out to test the effects of different culture media on the capture of paramecia. It was thought that the bladders might be stimulated to greater activity by media containing nutrient materials. The contrasting media used in these experiments were as follows (in every case the number of paramecia in the medium was increased by centrifuging the hay infusion, and the bladders were attached to branches of the plant): Exp. 29, spring water v.s. hay infusion; Exp. 38, tap water v.s. hay infusion v.s. beef meal v.s. malted milk v.s. 0.7% saline; and Exp. 41, 42, 44 and 46, hay infusion v.s. tap water after washing in tap water.

Experiment 29.—In hay infusion, 20 of 69 bladders, or 29 per cent., captured 35 specimens in a period of 2 hours; the number per positive bladder ranged from one to 86, and the average per positive bladder was 1.75. In spring water, 62 of 73 bladders, or 85 per cent., captured 272 specimens in the same period; number per positive bladder, one to 19; average, 4.40.

Experiment 38.—In hay infusion, 13 of 40 bladders, or 32 per cent., captured 23 specimens or 1.8 per positive bladder.

In tap water, 5 of 47 bladders, or 11 per cent., captured 5 specimens or 1 per positive bladder.

In 0.5 per cent. beef meal solution, 17 of 44 bladders, or 39 per cent. captured 21 specimens or 1.2 per positive bladder.

In 1.0 per cent. malted milk solution, 16 of 44 bladders, or 36 per cent., captured 27 specimens or 1.7 per positive bladder.

In 0.7 per cent. saline solution, 2 of 43 bladders, or 5 per cent., captured 2 specimens or 1.0 per positive bladder.

Experiments 41–46.—In hay infusion, 9 of 30 bladders captured 10 specimens; 4 of 30 captured 4; 19 of 61 captured 24; and 8 of 30 captured 10, respectively. Totals: 40 of 151 bladders captured 48 specimens; 26 per cent. of the bladders captured an average of 1.2 specimens per bladder.

In tap water, 12 of 30 bladders captured 16 specimens; 10 of 30 bladders captured 12; 26 of 66 captured 31; and 6 of 30 captured 8, respectively. Totals: 54 of 156 bladders captured 67 specimens; 35 per cent. of the bladders captured an average of 1.3 specimens per bladder.

From these results tap water seems to be a more effective medium for the capture of paramecia than hay infusion; 44 per cent. or 121 of 276 bladders captured 344 or 2.8 per bladder in tap water, whereas only 28 per cent. or 73 of 260 bladders captured 1.5 per bladder in hay infusion. The other media used were not sufficiently studied to give significant results, but saline solution is apparently disadvantageous, and beef meal solution and malted milk solution are as favorable as hay infusion or even more favorable, but not as effective as tap water. Observations of the activities of the paramecia in different media revealed the fact that the organisms swam about more rapidly in tap water than in any other medium. This means that in tap water the chances that paramecia will come in contact with the valves of the bladders more frequently and with more force are greater than in the other media and would lead to a larger number being captured in a given period. The conclusion based on these experiments is therefore that the differences in the number of specimens captured are due to the effects of the media on the rate of speed of the paramecia rather than their effect on the bladders.

Paramecia are killed by bladders, on the average, in a period of about 75 minutes. That paramecia die after being captured by utricularia bladders was proven by Exp. 1 described above. Considerable effort was made to determine the exact length of time required to bring about their death. Data from two types of experiments are available. In 16 experiments stems bearing bladders were placed in cultures containing paramecia and removed usually at the end of 2 hours but sometimes in one hour or in 4 hours. An immediate examination provided data regarding the number captured during this period and the numbers that had been killed or were still alive. The total number captured in these 16 experiments was 780 of which 563 were dead and 217 were alive when the bladders were examined. A summary of the data is as follows:

9 of 21 or 43 per cent. were dead one hour after the beginning of the experiments.
12 of 21 or 57 per cent. were alive one hour after the beginning of the experiments.
416 of 603 or 69 per cent. were dead two hours after the beginning of the experiments.
187 of 603 or 31 per cent. were alive two hours after the beginning of the experiments.
138 of 156 or 88 per cent. were dead four hours after the beginning of the experiments.
18 of 156 or 12 per cent. were alive four hours after the beginning of the experiments.

These data show that the percentage of paramecia killed increases from 43 one hour after the beginning of the experiments to 88 four hours after the beginning of the experiments. The exact length of time necessary to kill these protozoa could not of course be determined in this way.

Three other experiments were carried out in the following manner. Branches bearing bladders were immersed in a paramecium culture for a short period, then washed, placed under a large cover glass in a petri dish and flooded with water. Examinations of these preparations were made at frequent intervals.

Experiment 50.—A record of 9 captured paramecia shows one dead in 45 minutes; 2 active at least 42 minutes but dead in 56 minutes; one alive 49 min., but dead in 61 min.; one alive 54 min., but dead in 72 min.; one alive 64 min., but dead in 73 min.; one alive 75 min., but dead in 91 min.; one alive 84 min., but dead in 103 min.; one alive 104 min., but dead in 114 min. The average period during which these paramecia continued to live was 64 minutes and the average time until their death was 75 minutes.

Experiment 53.—In this experiment a record was kept of 23 captured paramecia. The period that these remained active after capture ranged from 20 to 127 minutes, with an average of 48 minutes, and the period until their death ranged from 34 to 141 minutes or an average of 72 minutes.

Experiment 54.—A record was made of 62 specimens in this experiment. Two were active 20 minutes; 3 from 30 to 40 min.; 5 from 40 to 50; 9 from 50 to 60; 15 from 60 to 70; 3 from 70 to 80; 9 from 80 to 90; 4 from 90 to 100; 3 from 100 to 110; and 9 from 113 to 170. Two were dead in from 20 to 30 minutes; 2 from 40 to 50 min.; 5 from 50 to 60; 7 from 60 to 70; 15 from 70 to 80; 5 from 80 to 90; 7 from 90 to 100; 2 from 100 to 110; 5 from 110 to 120; and 10 from 123 to 180. The active period of the greatest number of specimens was from 50 to 80 minutes, 35 of the 62 falling within this range. The average active period was 78 minutes. The death period of most of the specimens was from 50 to 98 minutes, 39 of the 62 falling within this range. The average death period was 93 minutes.

The average active period of the paramecia studied in Exps. 50, 53 and 54 was 64 minutes for 9 specimens; 48 minutes for 23; and 78 minutes for 62. If the data of these three experiments are combined the average active period after capture is found to be 69 minutes. The average period until death in the same experiments was 75 minutes for 9 specimens; 72 minutes for 23; and 93 minutes for 62. When the data of the experiments are combined the average period of the 94 spcimeens studied is found to be 85 minutes. It may be concluded that the actual death period lies between that when the specimens were last observed alive and that when they were first observed to be dead, which is on the average between 69 and 85 minutes. Seventy-five minutes may, therefore, be selected as approximately the length of time paramecia, on an average, remain alive after being captured by the bladders of utricularia.

When injected into attached bladders paramecia may die within a short period or remain alive for many days. In three experiments paramecia were injected with a fine pipette into bladders attached to branches of the utricularia plant.

Experiment 13.—Paramecia were injected into 6 bladders attached to one stem, from 2 to 10 specimens in each bladder. All were alive at the end of 2½ hours, but were dead in 3 of the bladders at the end of 26 hours. The paramecia remained alive in one bladder for 6 days and escaped probably because of the disintegration of the wall of the bladder. In another bladder two of the original 5 paramecia were still alive on the 16th day, but were very sluggish and quite transparent being evidently in a starved condition. One of these became abnormal in shape during its confinement; this specimen was still present after 17 days but was gone (escaped?) on the 18th day.

Experiment 52.—Nineteen bladders attached to 6 branches were injected with from one to 11 paramecia each. In three bladders the organisms died within 26 67 and 74 minutes respectively. In the other 16 bladders all the specimens remained alive at least 2 hours and in some of these the paramecia lived for 4, 5, 6 or 7 days.

Experiment 57.—Fifty-three bladders attached to 7 stems were injected with from one to 20 paramecia each. They were killed in 32 of the bladders within 4 hours as follows: in 3 bladders in 25, 51 and 55 minutes respectively; in 8 bladders within from one to 2 hours; in 11 bladders in from 2 to 3 hours; and in 10 bladders in from 3 to 4 hours. The paramecia disappeared (escaped?) from 5 bladders within 24 hours, but remained active in the remaining 16 bladders at least one day, and in 3 of them 2 days, in 3 of them 3 days, and in one of them 5 days.

When the results of these injection experiments are compared with those described in which the paramecia were captured by the bladders the most noticeable difference is the greater length of time required for the death of most of the organisms, extending as in Exp. 13 to a period of 17 days. This suggests that many of the injected bladders were not capable of bringing about the death of the paramecia and might not have captured specimens if placed in a culture medium containing them, or that the condition of the bladders was modified in some way during the process of injection. This subject will be referred to later.

When injected into detached bladders paramecia may die within a short period or remain alive for many days. Three experiments furnish data regarding the death of paramecia injected into detached bladders.

Experiment 11.—From 2 to 12 paramecia were injected into 4 bladders. They died in two bladders within 18 hours; escaped from one; and 9 specimens remained alive in the remaining bladder for 14 days; these paramecia were evidently suffering from lack of food. One of them remained alive for 16 days, but was gone (escaped?) on the 17th day.

Experiment M. 6.—Six bladders were injected with from one to 6 paramecia each. All were alive at the end of 1½ hours; all died within 6 hours except those in one bladder which died within 22 hours.

Experiment M. 12.—Fifteen bladders were injected with paramecia. They died in 10 bladders within 2 hours; in the other five they lived at least 2 days in 2, 3 days in 2, and 5 days in one.

These results are similar to those obtained by injecting paramecia into attached bladders and indicate that the separation of bladders from the plant has no effect upon their relation to the prey they capture.

Paramecia captured by or injected into attached bladders that have been irrigated with water usually die within several hours or live for several days. The bladders were irrigated by first inserting a fine pipette through the entrance and sucking out the contents. Further suction was then applied which drew a continuous stream of water into the bladder from the outside and up into the pipette. The inside of the bladders was thus thoroughly washed out and the bladders filled with fresh water.

Experiment 58.—Thirty-eight bladders on 5 branches were irrigated, placed in a paramecium culture for 1½ hours and then examined. Four bladders captured paramecia. Bladder *a* contained 2 dead and one alive; *b*, 2 alive; *c*, 4 dead and 4 alive; *d*, one alive.

Experiment 62.—Thirty-four bladders on 5 branches were irrigated, placed in a paramecium culture for 2 hours and then examined. Five bladders were positive. The paramecia escaped from 2 of the bladders; they were dead in 2 and alive in one.

Experiment 63.—Only one of 39 bladders on 7 branches became infected with paramecia; two specimens in this bladder died within 2 hours.

Experiment 64.—Only-one of 31 bladders on 5 branches became infected; the two specimens in this bladder died within 2 hours.

Experiment M. 11.—Six bladders on each of 4 branches were irrigated and then inoculated with paramecia. All were alive at the end of 1½ hours. After 9 hours the paramecia were dead in 19 bladders, alive in 4, and had escaped through a break in the wall of one. At the end of 24 hours the paramecia were dead in 22 and alive in one bladder.

Experiment M. 13.—Six bladders on each of 4 branches were irrigated and then inoculated with paramecia. All were alive after 2½ hours. At the end of 4½ hours all were alive in the bladders on branch *d*, all were dead on branch *b*, and 5 were dead and one alive on branches *a* and *c*.

Experiment M. 17.—Four branches bearing 6 bladders each were used. Bladders 1, 2, and 3 on each branch were irrigated and bladders 2, 4, and 6 were not; all were inoculated with paramecia. All were alive at the end of one hour. After 2½ hours all were dead on branch *b*; all were alive on branch *d*; all were dead in bladders 1, 3, 4 and 5 of branch *a* and in 4 and 6 of branch *c*. Paramecia remained alive for 54 hours in bladder 6 of branch *a*, in bladders 1, 2, and 3 of branch *c*, and in bladders 1, 3, and 5 of branch *d*. These all died within the next 4 days.

These experiments indicate that irrigated bladders do not succeed well in capturing paramecia but that the specimens captured usually die within 2 hours. Their failure to capture paramecia is due probably to the fact that many irrigated bladders do not take on the compressed condition necessary for capturing their prey (see page 243). Exps. M. 11 and M. 13 prove that paramecia inoculated into irrigated bladders usually live for several hours but that many of them die within from 4½ to 9 hours. Differences in the physiological condition of the different branches seems to be responsible for the fact that the paramecia die in all the bladders on one branch but remain alive in all those on another, as in Exp. M. 13 branches *d* and *b*. In Exp. M. 17 a critical experiment of irrigated and non-irrigated bladders is made. Very little difference is noticeable at the end of 2½ hours at which time the paramecia were dead in 6 irrigated and in only 2 non-irrigated bladders. The data indicate that irrigation delays but does not prevent the death of the paramecia.

Paramecia break down into a granular mass in attached or detached bladders that have captured them or into which they have been inoculated either with or without irrigation. The paramecia that die in the bladders of utricularia undergo disintegration in several different ways. Some of them dissolve into a mass of granules almost at once, spreading out as though an explosion had occurred within; others become opaque and abnormal in shape and then begin to break down at one or two points usually on the side; and still others although opaque and abnormal in shape retain their identity.

Experiment 52.—The paramecia in this experiment disintegrated into a mass of granules in the attached bladders that had been inoculated with them within 26 minutes, 24 hours, 2 days, 4 days and 5 days respectively.

Experiment 53.—The paramecia became granular in attached bladders that had captured them within the following number of minutes: 38, 68, 70, 83, 85, 123, 124, 127, 194. In at least 9 bladders they remained intact for 18 hours.

Experiment 54.—The paramecia became granular in attached bladders that had captured them within the following number of minutes: 27, 70, 71, 125. Five became granular within 2½ hours, and 8 within 20 hours.

Experiment 55.—Two specimens became granular in attached bladders that had captured them within 2 hours, 2 within 2¼ hours, and 12 within 2½ hours. Fifty-five were still intact at the end of 2½ hours.

Experiment 57.—The paramecia became granular in attached bladders that had been inoculated with them as follows: in 10 bladders within 18 hours; in 16 bladders within 24 hours; and in 8 within 48 hours.

Experiment 56.—The paramecia became granular in detached bladders that had captured them as follows: 6 within 40 to 58 minutes; 12 within 64 to 95 minutes; and 2 within 2½ hours. One was still alive and 143 were dead but not granular at the end of 4 hours.

Experiment 58.—Paramecia in this experiment were captured by detached irrigated bladders. Seven hours later 3 specimens had become granular, 11 were dead but not yet broken down and one was still active.

Experiment M. 13.—Paramecia were inoculated into attached irrigated bladders. No accurate record was kept but many specimens were granular at the end of 5 hours.

This series of experiments indicate that paramecia break down into a mass of granules under various conditions, (1) in attached bladders that have captured them, (2) in detached bladders that have captured them, (3) in attached bladders inoculated with them, (4) in attached irrigated bladders inoculated with them, and (5) in detached irrigated bladders that have captured them. The principal differences seem to be the length of time required to reach the granular state and the comparative number that become granular within a brief period, *e.g.*, within one hour or 2½ hours. A larger number of paramecia became granular in a shorter length of time within bladders that were attached or detached and had captured them (Exps. 53, 54, 55, 56) than in bladders into which they had been inoculated. No differences are apparent between attached and detached bladders. From the data available there appears to be no striking difference between bladders that have been inoculated with paramecia, attached or detached, irrigated or not irrigated.

Paramecia are captured more frequently by older, larger bladders than by younger, smaller bladders. In some of the earlier experiments the younger, smaller bladders seemed to become more readily infected with paramecia than those older and larger. Three experiments were carried out to test this. Branches bearing both young and old bladders were selected and the sections with the young and those with the old bladders cut out and placed in different paramecia cultures for 2 hours.

Experiment 39.—Three of 48 young and 6 of 48 old bladders became infected.

Experiment 40.—Eight of 30 young and 17 of 30 old bladders became infected.

Experiment 43.—Thirteen of 30 young and 11 of 30 old bladders became infected.

A total of 24 young and 34 old bladders became infected in these experiments. This is probably too small a number to be significant, but could be explained by the greater chance the older bladders offer because of the larger size of the entrance.

Paramecia do not die in a short time in old dead bladders or in mature bladders that have been killed by heat. When bladders become very old they loose their chlorophyll, become detached from the branches and fall to the bottom. Two experiments were carried out to determine whether paramecia are captured by these old, dead bladders.

Experiment 49.—Forty old bladders were placed in a paramecium culture for 2 hours. Seven of them captured specimens; none of these died and all had escaped within 24 hours, indicating that they had entered through openings in the walls of the bladders.

Experiment 65.—Sixty old bladders were similarly tested. No paramecia were captured.

Two experiments were carried out with mature bladders which were killed by being subjected to boiling water for 2 minutes.

Experiment 59.—Seven branches bearing 68 bladders were immersed in a paramecium culture for 1½ hours. All bladders were negative.

Experiment 61.—Nineteen bladders on 5 branches were inoculated with paramecia and kept under continuous observation for 2½ hours. None died. Twenty-four hours later many had escaped.

No differences in the behavior of paramecia were noted when placed in liquid from non-infected and infected bladders. Eleven experiments were carried out to determine whether any differences as regards the effect upon paramecium could be detected between the liquid from unfed bladders and that from bladders that had recently captured and killed paramecia. Liquid from the latter, as noted above (p. 254) has no effect on euglenas added to it.

Experiments 31, M. 8, M. 9, M. 14.—In these experiments the liquid from 107 bladders that had not captured paramecia was either squeezed out or withdrawn with a fine pipette, and either placed in a hollow ground slide in a moist chamber or treated as a hanging drop. Several paramecia were added to each and the preparations examined at frequent intervals. In Experiments M. 8 and M. 14 the paramecia were normally active at the end of 48 hours when the experiments were discontinued; in the other experiments the paramecia lived at least 5½ hours and death was later due to drying or to insufficient medium.

Experiments 26, 32, 60, M. 10 and M. 16.—Liquid from 203 control bladders and from 203 bladders that had killed paramecia was squeezed out or withdrawn with a fine pipette and its effects on paramecia tested. No significant difference was noted between the behavior of the paramecia in the liquid from non-infected bladders and in that from infected bladders. When a small amount of liquid was used the paramecia died in all cases within a few hours; when a larger amount was used no change was noted within 48 hours and the experiments were therefore discontinued.

The results of the experiments on paramecia described above are conclusive so far as they concern the effects of their capture or injection into the bladders under various conditions. Just how the results noted are brought about has not been determined and evidently will require for its solution extended experimental research. Among the questions that arise are the following:

What differences are responsible for the death of certain protozoa such as paramecium and stentor (see below) and the apparent ability of others such as euglena, heteronema, and phacus to live indefinitely within the bladders? (see below).

What is responsible for the rapid death of paramecia in certain bladders and their ability to live for many days in others?

Does the plant, when in a certain physiological state, secrete something into the bladder that brings about the death of paramecia?

Why do some paramecia disintegrate almost immediately and others from several hours to several days after death?

When several paramecia are confined in a single bladder, why do some disintegrate before others, as sometimes happens?

Is the same factor that is responsible for the death of paramecium also responsible for its disintegration?

Does the plant secrete something into the bladder that digests the paramecia?

It would be unprofitable to discuss these questions on the basis of the data available but certain observations not emphasized above have a bearing on them. In the first place, it is evident that paramecia are not killed quickly by confinement, since in some of the experiments specimens lived for several weeks in the bladders. The physiological state of the plant apparently has a large influence on the paramecia. For example, in Experiment 57 all the paramecia injected into bladders on one stem died within a few hours and all injected into those on another stem were all alive at the end of 24 hours. In another case (Experiment M. 13) paramecia were injected into 6 irrigated bladders on each of 4 branches; they were all dead in the bladders on three of the branches within 5 hours but remained alive in certain of the bladders on the fourth branch for 8 days. These experiments indicate that the plant when in the proper physiological condition secretes something into the bladders that kills paramecia but at other times simply confines the organisms within the bladders. Attempts were made to determine whether or not the plant exhibited evidences of hunger and satiety by examining bladders continuously over a period of several days, but the results are not significant. Another problem considered was the possible selection of organisms by the bladders. No experimental data are available on this point but the observations on the types of organisms captured and on the method of capture led to the conclusion that any organism may be captured that is small enough to enter the vestibule of the bladder and actively moves about within the vestibule.

The best tests for the presence of digestive enzymes within the bladder have been reported by von Luetzelburg (1910) who comes to the conclusion that the organisms captured by the bladders are utilized as food by the plant by means of an enzyme acting in an alkaline medium. An admixture of acid (benzoic acid) prevents the organisms from injuring the walls of the bladder. Digestion within the bladder is slow but thorough.

The valve of the bladder is closed so tightly that nothing within can escape.

At present there is little doubt regarding the utilization by the plant of material derived from the animals captured and digested. Darwin concluded from a study of the quadrifid cells in the inner walls of the bladders before and after the capture and disintegration of food that the plant absorbed nutriment derived from the captured animals. Further evidence that this is true is offered by Büsgen (1888) who measured fed and unfed plants at intervals during a period of about three weeks. The fed plants grew twice as fast as the unfed plants during this period, apparently because of the nutriment supplied by the animals captured and absorbed by the bladders of the latter.

Experiments with Other Protozoa.

Although the principal experiments were carried out with paramecia and euglenas a few other protozoa were used as opportunity offered. These included representatives of all three classes of the free-living Protozoa—Sarcodina, Mastigophora and Infusoria.

Centropyxis aculeata is captured by bladders and starved to death. Shells of specimens of this species were observed in bladders brought into the laboratory, but these apparently contained no living animals. Two experiments were performed to determine the relations of this species to the plant.

Experiment 37.—Fifteen detached bladders were placed in a concavity in water containing a number of large specimens of *C. aculeata*. Six days later 6 of these bladders were found to be infected, 4 with one and 2 with 2 each. The shells contained very little protoplasm—approximately one tenth of the original amount—but this was still alive and able to form pseudopodia. Apparently the conditions in the bladder have no directly injurious effect upon these rhizopods but deprive them of opportunities for capturing food and hence lead to their starvation.

Experiment 27.—Four bladders on one stem were inoculated, three with one and one with 2 specimens of *C. aculeata*. Seven days later they were dissected out and examined. Two shells were empty and the other three contained a very small amount of living protoplasm. The former apparently were starved to death and the latter were almost dead of starvation.

Heteronema acus is captured but not injured by bladders. This green flagellate was used in 2 experiments.

Experiment 16.—Four bladders, 2 on each of 2 stems were inoculated with several specimens each. These bladders soon died and disintegrated but one of them still contained active specimens 3 days later.

Experiment 18.—Three stems bearing 7, 4 and 3 bladders respectively were placed in a culture containing *H. acus.* Twenty-four hours later specimens had been captured by 2 bladders on the first stem, 3 on the second and 3 on the third. Daily examinations were made for 5 days. During this time the flagellates retained their normal activities but apparently did not increase in numbers.

Phacus longicaudus is not injured by bladders and may multiply in bladders.

Experiment 28.—Twelve bladders on three stems were inoculated with from one to 7 specimens each of *P. longicaudus.* These specimens retained their normal appearance and activities. At the end of 7 days an increase in the number of specimens was noted in at least 5 bladders; these contained 6, 7, 8, 11, and 25 specimens each.

Stentor polymorphus is captured and killed by bladders.

Experiment M. 1.—One stem bearing 8 large and many smaller bladders was placed in a culture containing many specimens of *S. polymorphus.* Twenty-two hours later one bladder had captured 2 and another bladder one specimen. Two of the stentors were still alive with cilia moving but were abnormal in shape, the third had become spherical and quiescent.

Experiment M. 2.—Seven detached bladders were inoculated with stentors; 2 with one, 3 with 3, one with 4, and one with 6 specimens. Eighteen hours later the stentors in 3 of these bladders (7 stentors in all) had died and disintegrated; those in 2 bladders (5 specimens) were still active; and those in 2 bladders (9 specimens) were not active and abnormal in shape. One stentor remained active for 42 hours but all were dead and had disintegrated by the end of 66 hours.

Stylonychia pustulata is captured and killed by bladders.

Experiment M. 5.—Twelve large detached bladders were placed in a culture containing many specimens. Four hours later 5 had captured 1, 2, 2, 3 and 4 specimens respectively. These had all disappeared 20 hours later.

Experiment M. 3.—Eleven large detached bladders were inoculated each with a few specimens. Some of these were still alive and active 3 hours later. All but 2 specimens in one of the bladders had disappeared 20 hours later.

Experiment M. 4.—Eleven large detached bladders were inoculated with from one to 10 specimens each. Four hours later active specimens were noted in only 5 bladders, and 20 hours later only one specimen was found in one of them.

Colpidium colpoda is captured and may live from 7 to 24 hours or more. In seven experiments the culture used contained not only paramecia or stylonychias but also *C. colpoda.* The reactions of the latter were incidentally recorded.

Experiment M. 3.—Specimens of Colpidium were still alive in 8 of 11 detached bladders that had been inoculated 23 hours previously.

Experiment M. 4.—Specimens were alive and active in all cases 24 hours after inoculated into 11 detached bladders.

Experiment M. 7.—Colpidia were captured by all of 19 detached bladders but had disappeared in all but 2 seven hours later.

Experiment M. 11.—Colpidia were inoculated into 24 attached bladders (6 on each of 4 stems) that had previously been irrigated. They were active in at least 8 of these bladders 23 hours later.

Experiment M. 8.—Colpidia lived at least 48 hours in liquid drawn from 15 bladders and kept in a moist chamber in a hollow ground slide.

Experiment M. 10.—Colpidia lived at least 44 hours in liquid drawn from 15 bladders that had recently killed paramecia.

Chilomonas paramecium is captured by bladders. The medium used in Experiments 10, 24, and 25 contained many specimens of *C. paramecium* as well as paramecia, and it was noted that many of both the attached and detached bladders captured them. In some cases they may have entered at the same time paramecia were taken in but they were also present in bladders that had not captured paramecia.

Material containing intestinal flagellates of tadpoles quickly kills bladders. Four attempts were made (Experiments 3, 14, 21 and 22) to determine whether *Trichomonas augusta*, *Hexamitus batrachorum*, and *Euglenamorpha hegneri* from the intestine of tadpoles would be captured by and live in the bladders. Bladders were immersed in diluted intestinal contents containing these flagellates, and both attached and detached bladders were inoculated with them, but the bladders were quickly killed hence no definite results were obtained.

Nematodes and rotifers are captured by and live in bladders for many hours.

Experiment 4.—Several stems were immersed in a culture containing both euglenas and free-living nematodes; two days later 7 of the 13 bladders contained from 1 to 4 or more nematodes most of which were active.

Active rotifers were found in many of the bladders recently brought into the laboratory. These remained active for several days indicating that they are able to withstand conditions within the bladders for a considerable period.

Summary and Conclusions.

Summary. 1. The bladders (utricles) of *Utricularia vulgaris* v. *americana* Gray capture large numbers of small free-swimming aquatic organisms; those on parts of a plant 220 cm. long contained approximately 150,000 entomostraca and many other organisms.

2. Living protozoa of various kinds were found in the bladders; these were principally euglenas, but a few other flagellates, minute ciliates, and amœbæ were encountered.

3. Animals do not force their way into the bladders as hitherto supposed but are captured as follows: the normal bladder has compressed side walls; organisms enter the vestibule and by their movements stimulate the valve to open; water rushes in as the side walls expand and sucks in with it the stimulating organism; then the valve closes and the organism is unable to escape.

4. When a bladder expands its contents are increased approximately 88 per cent. by the outside medium which is sucked in with the prey.

5. Bladders expand when the wall is punctured and when the mouth opening is laterally or antero-posteriorly compressed.

6. Expanded bladders recover their compressed condition and are "set" for another capture in about 20 minutes. How 88 per cent. of their contents pass out during the period of compression is not known.

7. Euglenas occur frequently in bladders in nature but plants loose their infection if kept for many months in the laboratory. Free-living euglenas are captured by bladders and live and multiply within. They are not injured in bladders that have recently captured and killed paramecia. The euglenas that inhabit bladders in nature are probably captured free-living species and not species peculiar to the utricularia plant.

8. Paramecia are captured by bladders that are either attached to or detached from the plant; about 30 per cent. of bladders immersed in paramecium cultures succeeded in capturing one or more specimens within an hour. The number of paramecia captured by the bladders depends upon the culture medium only in so far as this affects the rate of swimming of the organisms and hence their chances of coming in contact with the valve of the bladders. Paramecia die within the bladders in an average period of about 75 minutes; some of them lived for many hours and some that were introduced into bladders with a fine pipette lived for 17 days. Paramecia die usually within several hours in bladders previously thoroughly cleaned out by irrigation.

Paramecia usually break up into granules within several hours after capture. Paramecia are not killed in old dead bladders nor in mature bladders killed by heat.

9. Specimens of the shelled rhizopod, *Centropyxis aculeata*, may be captured by bladders; they are not directly killed but die slowly of starvation.

10. The green flagellate, *Heteronema acus*, is captured but not injured by the bladders. *Phacus longicaudus*, is captured by bladders but not injured; it may multiply within the bladders. Bladders capture *Chilomonas paramecium*.

11. The ciliate, *Stentor polymorphus*, is captured and killed by bladders as is also *Stylonychia pustulata*. *Colpidium colpoda* is captured but may live for a day or more.

12. Material from the intestine of tadpoles containing flagellates was introduced into bladders, but the bladders were quickly killed.

13. Nematodes and rotifers captured by bladders live for many hours within.

Conclusions.—The principal conclusions from these studies are, (1) organisms do not force their way into bladders but are captured by them; (2) the bladders do not select their prey but any organism small enough to enter the vestibule may be captured; (3) organisms as small as *Chilomonas paramecium* (24 microns in length) may be captured; (4) the protozoa found in the bladders are not intruders but captives and are probably all free-living species; and (5) some of the captured protozoa live successfully within (*Euglena*, *Heteronema*, *Phacus*), some slowly starve to death (*Centropyxis*) and others are more or less quickly killed and digested (*Paramecium*, *Stentor*, *Stylonychia*, *Colpidium*). The data presented indicate that the bladders secrete something that kills and digests paramecia but does not injure euglenas. The origin and character of this secretion and the changes in the plant during the compression and expansion of the bladders are problems for the plant physiologist to solve.

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THE PROTOZOA OF THE PITCHER PLANT, *SARRACENIA PURPUREA*.[1]

R. W. HEGNER.

Investigations of protozoa within the bladders of utricularia plants led the writer (Hegner, 1926) to examine the literature on other insectivorous plants and to carry out the experiments described below. The general problem under attack is that of host-parasite relationships and the specific problem is that of the relations between intestinal protozoa and their environment. Primitive conditions of digestion might be expected in insectivorous plants and this idea is responsible for the present investigation. Whether or not the liquid in the pitchers of the pitcher plants contains digestive enzymes secreted by the plant, which digest insects and other animals that enter the pitcher, is a problem that has been carefully studied by many investigators for many years. On several occasions when the problem seemed to be solved, a new factor was suggested that made it necessary to carry on further investigations. Among the most recent papers is that of Hepburn (1922) who concludes "on both morphological and bio-chemical grounds, that the pitchers of most, if not all, species of pitcher plants are designed for the capture and digestion of prey as a means of nutrition for the plant" (page 776). Among the species studied by Hepburn, St. John and Jones (1920) was *Sarracenia purpurea* and proteolytic enzymes (proteases) were found in the pitcher liquid of this species. Many types of experiments have been used to determine the character of the pitcher liquid but no one seems to have employed protozoa as indicators.

That living protozoa occur in the pitcher liquid of various species of pitcher plants has been mentioned by several investigators but only one worker (van Oye, 1921) has made a careful study of the species. In the pitcher liquid of *Nepenthes melam-*

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phora, van Oye noted 7 species of rhizopods, all shelled except 2 species of amœbæ. He identified these as *Centropyxis aculeata*, *Difflugia constricta*, *Lesquereusia epistomium*, *Arcella vulgaris*, *Cochliopodium bilimbosum*, *Amœba guttula*, and *Amœba nepenthesi*. *Amœba nepenthesi* is a new species named by van Oye. It is very small, with short, blunt pseudopodia and a body more or less egg-shaped. Cysts of it were found which soon opened when placed in a drop of water or in liquid from another plant, and liberated the amœba within.

Protozoa are present in the pitcher liquid of *Sarracenia purpurea* in nature. My examinations give only a general idea of the protozoan fauna of the pitchers. Several pitchers were examined from plants in the greenhouse of the university and ten pitchers were examined from plants obtained at Salisbury Cove, Maine. Organisms were looked for first in a sample of the liquid with a binocular microscope; then all of the liquid was centrifuged and the contents at the bottom of the centrifuge tube was studied with a compound microscope; and finally the debris at the bottom of the pitcher was examined. No attempt was made to identify definitely the various organisms encountered. Amœbæ, flagellates and small ciliates were found in the pitchers from Baltimore. Ten pitchers from Maine were taken from different plants growing in separate localities. They contained the following organisms.

1. Young pitcher containing 9 cc. of milky liquid. Protozoa, few small flagellates resembling *Cercomonas crassicauda;* insects, many ants, one house fly (?); insect larvæ, living, many; mites, one; rotifers, few.
2. Young pitcher containing 11 cc. of clear liquid. Protozoa, few cercomonas-like flagellates; insects, many, badly broken up; insect larvæ, living, several.
3. Mature pitcher containing 15 cc. of clear liquid. Protozoa, few cercomonas-like flagellates, several ridged flagellates, several radiosa-like amœbæ; insects, many, badly decayed; insect larvæ, several; mites, one; rotifers, few; nematodes, many.
4. Mature pitcher containing 7 cc. of milky liquid. Protozoa, many cercomonas-like flagellates; insects, many ants, one

house fly (?), one spider, one lace-wing fly; insect larvæ, living, many; mites, few; rotifers, many.

5. Mature pitcher containing 8 cc. of cloudy liquid. Protozoa, several varieties of cercomonas-like flagellates, many ridged flagellates, many amœbulæ, several limax amœbæ, several actimospherium-like rhizopods, many prorodon-like ciliates and many holosticha-like ciliates; insects, few, badly decayed; insect larvæ, living, many; rotifers, many; nematodes, many; entomostraca, few.
6. Mature pitcher containing 12 cc. of clear liquid. Protozoa, several cercomonas-like flagellates, many ridged flagellates, one amœbula; insects, few, badly decayed; insect larvæ, one; rotifers, many; entomostraca, one.
7. Young pitcher containing 17 cc. of clear liquid. Protozoa, many cercomonas-like flagellates; insects, few; insect larvæ, many; mites, few; rotifers, few.
8. Young pitcher containing 25 cc. of milky liquid. Protozoa, few bodo-like flagellates, few amœbulæ, many holosticha-like ciliates; insects, few; insect larvæ, many; mites, few; rotifers, few.
9. Young pitcher containing 19 cc. of clear liquid. Protozoa, many amœbulæ, many bodo-like flagellates; insects, many; insect larvæ, many.
10. Young pitcher containing 17 cc. of clear liquid. Protozoa, many cercomonas-like flagellates, few mastigamœba-like flagellates, few bodo-like flagellates; insects, few; insect larvæ, few; mites, one; rotifers, few.

Protozoa were present, as noted, in all ten pitchers and all three classes of free-living protozoa were represented by several species. The liquid from many other pitchers was examined in the experiments described below and similar protozoa were found in most of them and probably would have been found in all if a more careful search had been made. The most common living organisms encountered in the pitchers were insect larvæ, mites and rotifers. Nematodes and entomostraca were found in a few of the pitchers, and tardigradas in one.

How does the pitcher liquor become populated with protozoa? So far as I know no experimental data on this point exist. The protozoa found living in the pitchers may be of two types, (1) free-living species and (2) species adapted for life in the pitcher liquid and restricted to this habitat. The pitchers are visited by many insects which are drowned in the liquid and these no doubt are frequently soiled with material containing cysts or even living trophozoites of free-living protozoa. This seems to be a simple avenue for the entrance of free-living species. Species that may be peculiar to the pitchers may similarly be transferred from one pitcher to another by the insects that live in the pitcher plant liquid and which fly from pitcher to pitcher to lay their eggs.

Experimental Studies on Free-living Protozoa in Pitcher Liquid.—Paramecia were the principal protozoa used in these experiments; Colpoda and Chilomonas were present in some of the paramecium cultures. Four types of experiments were performed; (1) paramecia were introduced into pitcher liquid in hollow ground slides, (2) into open pitchers in the laboratory, (3) into unopened pitchers in the laboratory and (4) into open pitchers in the field.

1. Paramecia in pitcher liquid in hollow ground slides: In three experiments liquid from 24 pitchers on plants brought into the laboratory was used. Five drops of liquid from each pitcher were placed in one hollow ground cavity of a slide and one drop of culture medium containing paramecia was added; five drops of culture medium were placed in the other cavity and one drop containing paramecia added. All the preparations were examined at frequent intervals during the first two days but after that daily for from 5 to 7 days. In every preparation the paramecia lived as well in the pitcher liquid as in the culture medium, and although no exact count was made, it was evident that multiplication occurred in at least 8 of the samples of pitcher liquid.

2. Paramecia in open pitchers in the laboratory: Paramecia were introduced into the liquid in 22 pitchers on four plants in the laboratory. The liquid in these pitchers was present when the plants were brought in. Eight of the pitchers were old, 8 mature, and 6 young. Samples were taken from these pitchers daily during the succeeding 5 days. Paramecia were recovered

from 15 of the 22 pitchers during this period. They may have been present in all, since it was discovered later that the paramecia tend to congregate at the bottom of the pitcher where the liquid is rich in organic debris; whereas the samples were taken from the liquid near the top. There was an evident increase in the number of paramecia present in at least 3 of the pitchers. Colpoda was recovered from 11 pitchers and apparently had multiplied in 4 of these.

3. Paramecium in unopened pitchers in the laboratory: Several cc. of culture medium containing paramecia were introduced into each of 4 unopened pitchers and the lips of the pitchers fastened together to prevent the entrance of insects. Samples were examined from these daily for 12 days. In 2 of the pitchers paramecium, and also colpoda and chilomonas, were more abundant at the end of the experiment than at the beginning; in the third pitcher paramecium was present in about the same numbers as at first; colpoda and chilomonas had increased; and in the fourth pitcher paramecia were present on the tenth day but absent on the eleventh and twelfth days; colpoda decreased in numbers but chilomonas had increased.

4. Paramecium in open pitchers in the field: In order to avoid the possibility that changes might occur in the pitcher liquid when plants are brought into the laboratory, paramecia were introduced into ten pitchers borne by 5 plants in the field. These were labelled and examined on the following day and 2 days later. Paramecia were present in only 2 samples taken after 24 hours, colpoda in 7 and chilomonas in 7. On the second day the pitchers were brought into the laboratory, except one which had lost its label and could not be identified, and paramecia were recovered in all 9 of them. Apparently no decrease in numbers had occurred in the 48 hours of the experiment and increases seemed probable in several pitchers. The reason paramecia were found on the first examination in only 2 pitchers is that the organisms had congregated near the bottom of the pitcher where food conditions were favorable and, therefore, could not be recovered from the samples which were taken near the surface. Colpoda and chilomonas were recovered also from all 9 pitchers with no apparent decrease in numbers.

It was a surprise to find that paramecia are able to withstand the digestive enzymes in the pitcher liquid. There may still be some question as to whether these enzymes are produced by the plant or by bacteria, but their presence is not generally disputed by investigators. According to Dr. Edgar T. Wherry (in letter) the pitcher liquid varies widely in reaction when the hydrogen-ion concentration is measured so that the paramecia in my experiments were probably living in solutions that differed considerably in this respect. That paramecia are able to withstand conditions within the digestive tract of frogs has been shown by Cleveland (MS). Rich cultures of paramecia were injected into the rectum of living frogs; in some cases one or less per cent. of them became encysted within usually from one to 2 hours; the rest were killed. In one instance encystment occurred within 30 minutes.

So far as the problem is concerned that the experiments described above were designed to solve, the conclusions seem justified that, (1) paramecia are able to live within the liquid either outside or within the pitchers of various ages borne by vigorous plants for an indefinite period without injury; (2) paramecia may multiply in the pitcher liquid; (3) colpoda and chilomonas are similar to paramecia in these respects; (4) the protozoa that occur in nature in the pitcher liquid are probably free-living species carried there by flies although some of them may be species restricted to this particular environment.

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BIOLOGICAL BULLETIN

SPERMATOGENESIS OF THE BLACK-CLAWED CRAB, *LOPHOPANOPEUS BELLUS* (STIMPSON) RATHBUN.

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Introduction.

During the past few years I have been studying various cytological aspects of the gonads of numerous Pacific coast Brachyura (Fasten, '17, '18, '21 and '24) giving particular attention to the problem of spermatogenesis. The present paper is a continuation of the above research, dealing with the maturation of the male gametes of the black-clawed crab, *Lophopanopeus bellus* (Stimpson) Rathbun. This Brachyuran is a native of the Pacific coast of the United States, and according to Schmitt ('21), it ranges from Alaska to southern California. The material upon which the present contribution is based was obtained mainly along the shores of Puget Sound. In certain localities of this region, particularly in the San Juan islands, the black-clawed crab is found to be quite abundant.

Material and Methods.

The material used consisted of smear preparations of the testis of the crustacean under consideration. Although sectioned material was available, nevertheless the smears were found to be so superior that all of the stages and drawings were derived from them. The time for obtaining the best material was the latter part of June and the first portion of July. These periods agree quite well with what has been found to be the case in the testes of other Brachyura.

The smears were prepared in the usual manner, as described in my earlier paper on the spermatogenesis of the edible crab, *Cancer magister* Dana (Fasten, '18). Numerous fixatives were tried, but Flemming's strong solution was found to yield the best results. This mixture made the principal parts of the cell stand out clearly. Furthermore, it produced some differentiation in the staining capacities of the chromatoid bodies and the chromosomes. The former invariably stained less heavily than the latter. This was particularly noticeable when the slides were considerably destained. As for staining, the best results were obtained with Heidenhain's iron-alum hæmatoxylin and a counter-stain of acid-fuchsin.

The mature radial spermatozoa were studied in various isotonic, hypotonic and hypertonic solutions in order to discover the manner in which they function. These experiments have already been published in a separate paper (Fasten, 1921) and therefore the results will not be repeated here.

Description of Male Gonads.

The male reproductive organs of *Lophopanopeus bellus* lie in the cephalothoracic region. They consist of a bilobed tubular testis and a pair of vas deferent ducts. Each lobe of the testis runs laterally in the space between the heart and the digestive glands. Directly below the anterior portion of the heart the testicular lobes unite and from this junction point the convoluted vas deferent ducts originate. These run posteriorly to the base of the fifth pair of walking legs where they open to the outside.

The male gonads attain their maximum size during the latter part of June and the early portion of July. Between these times the cells in the tubules of the testis are undergoing rapid proliferation and all stages in the spermatogenesis process may be secured. Typical cross-sections of the testis reveal a similar picture to that given by me in 1918 for the edible crab, *Cancer magister* Dana. Some of the tubules contain just one typical stage; others show two distinct stages, while still others reveal three steps in the maturation process.

In the posterior region of the testis, where the lobes unite, and in the vas deferent ducts are found the mature spermatozoa. In *Lophopanopeus bellus*, the mature spermatozoa within the distal ends of the vas deferent ducts are surrounded by a single large spermatophore. This is contrary to what has been found in the other Brachyura studied, where numerous spermatophores are developed.

SPERMATOGENESIS.

A. Spermatogonial Stages.

In the black-clawed crab two distinct spermatogonial divisions can be seen (Figs. 1–2 and 8–11). The primary spermatogonial stages (Figs. 1–2) are considerably larger than those of the secondary spermatogonia (Figs. 8–11) but otherwise the division proceeds in the same manner as seen in Figs. 8–11. The resting primary spermatogonium (Fig. 1) is almost twice the size of the resting secondary spermatogonium (Fig. 8). Their structure, however, is similar. Numerous chromatin clumps and linin strands may be distinguished within the nucleus. A large nucleolus may also be recognized. The cytoplasm is uniform throughout, with the exception that occasionally larger masses that stain somewhat like chromatin may be seen. These masses are clearly discernible in Fig. 1. The centrosome which is a single granule is nearly always present.

When any one of the spermatogonia divides, the chromatin within the nucleus begins to fragment until a great many large, heavily staining clumps appear, as shown in Fig. 2. The nuclear wall soon breaks down with the result that the cell enters the metaphase stage of division (Fig. 9). Polar views of the metaphase showed the chromosomes to be rather small and numerous. Accurate counts could not be made although in many cases over one hundred of them were found distributed throughout the equatorial plane. But since it has been definitely determined that there are 62 bivalent chromosomes in the reduced number, there must be 124 univalent ones in the spermatogonial counts.

The anaphase (Fig. 10) and telophase (Fig. 11) stages follow quickly, thereby completing the spermatogonial divisions. The division of the secondary spermatogonia produces the resting primary spermatocytes (Fig. 12) which then continue the maturation process.

In connection with the spermatogonial stages reference must be made to the so-called "nutritive cells" (Figs. 3–7) which are commonly found associated with the spermatogonial strips located in tubules where there are mature spermatozoa. These so-called "nutritive cells" are huge structures assuming numerous shapes. Typical examples are pictured in Figs. 3–7. The delineation between the cells is oftentimes effaced, so that the nuclei seem to lie in a syncitial mass of cytoplasmic material. There is considerable difference of opinion amongst cytologists as to the probable relation between the nutritive and spermatogonial cells of decapod Crustacea. Grobben ('78), Gilson ('86) and Herrmann ('90) look upon the nutritive cells as foundation germinal cells from which the spermatogonia originate. In contrast to this view, such workers as St. George ('92), and Keppen ('06) regard the nutritive cells as being derived through a transformation of spermatogonia which have not undergone maturation. My own observations are in accord with the latter view. Such stages like Figs. 3 and 4 resemble primary spermatogonial cells (Fig. 1) but are larger in size.

Many of the workers on the cytology of the Decapoda have claimed that the nutritive cells undergo amitotic division. In sectioned material it seems as if this were the case. For instance certain sections through such cells as illustrated in Figs. 5 and 6 would yield typical examples for those advocating amitosis. However, when smears of the entire cells were obtained, the true condition is discovered. I have found numerous indications that mitosis is the normal process of division in these nutritive cells. A good review of the literature on this subject can be obtained in my earlier paper on the spermatogenesis of the American crayfish, *Cambarus virilis* (Fasten, '14).

B. Primary Spermatocyte Stages.

In the primary spermatocyte stages, the cells undergo growth, synapsis and reduction in the number of chromosomes. A single chromatoid body makes its appearance within the cytoplasm during the early stages of the growth period. The general sequence of events is quite similar to that found in other decapod Crustacea (Fasten, '14, '18, '24).

Growth Period.—The resting primary spermatocyte (Fig. 12) is slightly smaller than the resting secondary spermatogonium

(Fig. 8). Within its nucleus may be seen numerous irregular chromatin clumps distributed along a meshwork of thin strands. A single centrosome can also be distinguished.

When the primary spermatocyte begins its process of development, the first noticeable change occurs in the nucleus. The chromatin clumps fragment (Figs. 13 and 14) and soon weave out into a large number of leptotene threads (Fig. 15). These threads are distinct but so numerous that it was impossible to get any count of them.

The next step in the growth process is the pre-synaptic stage (Fig. 16) in which the leptotene threads wander to one pole of the nucleus and become lined up side by side into pairs. Within the cytoplasm a single, spherical chromatoid body makes its appearance and it is invariably surrounded by a clear area. This is quite similar to the bodies found by Wilson ('13), Komai ('20) and myself ('14, '18 and '24) for other forms. In slides which were considerably destained the chromatoid body took on a lighter hue than the chromatin of the nucleus. I found this same differentiation in destained preparations of *Cancer productus* Randall, *Cancer oregonensis* (Dana) Rathbun, and *Cancer gracilis* Dana which were fixed with Flemming's strong solution. From this stage on the chromatoid body persists and can be easily distinguished.

The pachytene stage follows (Fig. 17) in which synapsis of the paired leptotene threads takes place thereby transforming them into distinct gemini. In many of the preparations the line of fusion between some of the leptotene threads could be clearly seen. This is similar to the condition found in *Cambarus virilis* (Fasten '14), but differs from that found in the various Cancer crabs studied (Fasten, '18 and '24) where the fusion between the leptotene threads is complete.

The diplotene and post-diplotene stages (Figs. 18 and 19) follow each other in logical sequence. In the diplotene stage the paired threads of each geminus open up at one end along the longitudinal plane of fusion but remain attached at the opposite end, thereby producing figures like V, 8 and U. At the same time a second longitudinal furrow occurs along each of the opened arms, thus converting each geminus into four thin strands attached at one end, as seen in some of the V's, U's and 8's of Fig. 18.

The four components of each geminus continue to open up in such a manner as to produce typical X's like those shown in Fig. 19. This transformation marks the end of the post-diplotene stage and the cell is now ready to terminate the growth period by the formation of tetrads.

Figure 20 shows the pre-tetrad condition. The opposite ends of the X's soon wander towards each other and the middle fusion point becomes effaced. The result is that every one of the X's is converted into four thin strands, having the appearance of a pair of parallel lines with a transverse split in the middle.

Through a further condensation, each of the four thin strands is modified into a spherical chromosome, thus converting every geminus into a tetrad (Figs. 20 and 21). Following this, the typical bivalent dumb-bells are formed through a fusion of the pairs of univalent chromosomes of the tetrads. This is particularly well shown in Fig. 21. Here, also, the centrosome has divided into two elements and each is seen to migrate away from the other. At the same time the nuclear wall has begun to disintegrate and the cell, in general, has terminated its process of growth.

A careful study of the various stages of growth shows that the chromatoid body makes its appearance in the pre-synapsis period and persists from then on. A centrosome and an idiozome are clearly visible. As for synapsis, it occurs in parasynaptic fashion in which there is a side-by-side conjugation of the chromosomes. This is similar to what happens in the other decapod Crustacea which I have studied (Fasten, '14, '18 and '24). Komai ('20) has also found parasynapsis in the crustacean *Squilla oratoria* de Haan.

Reduction Division.—Following the disintegration of the nuclear wall, the primary spermatocyte enters the metaphase period (Figs. 22 and 23). The bivalent dumb-bells are lined up in the equatorial plane, and from them the delicate spindle fibres converge towards the centrosomes which are located at opposite poles. The single chromatoid body wanders undivided towards one of the poles of the cell. In some instances it lies amongst the spindle fibres (Fig. 22) while at other times it is located outside the spindle proper, as shown in Fig. 23. As already mentioned, when the slides were greatly destained, the chromatoid body could be easily distinguished from the chromosomes as it took on a lighter color. The most consistent counts of polar views of metaphase stages showed 62 bivalent chromosomes distributed throughout the plane of the equator (Fig. 24).

The anaphase and telophase stages (Figs. 25–28) follow quickly, ultimately producing the secondary spermatocytes. Figure 28 shows the two types of secondary spermatocytes formed, one containing the single chromatoid body, while the other is devoid of any such structure. These two kinds of secondary spermatocytes occur in equal number.

C. Secondary Spermatocyte Stages.

The secondary spermatocyte divisions are equational. No rest period is found after the mitosis of the primary spermatocyte, the cells immediately enter the metaphase to undergo rapid division. No good counts of the chromosomes of secondary spermatocytes could be made. The cells on the whole are almost half the size of the primary spermatocytes.

Figures 29–34 show the typical stages in the division of the secondary spermatocyte which possesses a chromatoid body, while Figs. 35–38 show the same process in the secondary spermatocyte that is minus the chromatoid body. The ultimate result of all these divisions is the formation of two kinds of spermatids, one which contains a chromatoid body (Fig. 39) and the other that lacks it (Fig. 44). This latter spermatid is about three times as numerous as the former one.

D. Transformations of Spermatids.

The two types of resting spermatids (Figs. 39 and 44) are spherical cells which have large masses of chromatin within the nuclei that stain intensely black with Heidenhain's hæmatoxylin. The cytoplasm is uniformly granular, possessing a distinct centrosome. The chromatoid body can be clearly seen within one of the kinds of spermatids (Fig. 39).

Transformations of the two spermatids occur in similar fashion.

The first change is a disintegration of the dense chromatin of the nucleus. This is consistently reduced until there are two spherical granules (Fig. 45) and then one (Figs. 40 and 41) left. These single granules appear like karyosomes. While all this is going on the nucleus takes on a lighter stain and at the same time, a mass of dense material makes its appearance in the cytoplasm which stains like chromatin (Figs. 40, 41 and 45). Koltzoff ('06) and Binford ('13) regard this material as mitochondria. I think this is nuclear material which has diffused out into the cytoplasm as the chromatin content has become reduced. I found a similar condition of affairs in the spermatids of *Cancer magister* (Fasten, '18).

Soon a vacuole makes its appearance at one end of the cytoplasm, the nucleus wanders towards the opposite pole and the centrosome with the mitochondria-like mass take the middle position between them (Figs. 42, 43, 46 and 47). In the spermatid which contains the chromatoid body, all changes go on as usual, but the chromatoid body wanders to the periphery of the cytoplasm and is ultimately eliminated from the cell (Figs. 39–43), thus playing no further part in the transformations. From now on the spermatids seem to be similar and the same changes take place in them.

The mitochondrial mass soon becomes ring shaped and the centrosome occupies its center. The upper end of the nucleus has penetrated the open inner space of the mitochondrial ring as seen in Figs. 48 and 49. The karyosome-like granule has also wandered upward until it comes to lie directly below the centrosome. The spermatid now appears like Fig. 49.

Subsequent to this stage there is a fusion between the centrosome and the karyosome-like body of the nucleus to form a single structure. This then elongates to look like a short rod (Fig. 50). Going hand in hand with these changes, a bubble makes its appearance at the upper end of the first vacuole (Fig. 50), which gradually increases in size (Figs. 50–55) and becomes the second vacuole. In *Lophopanopeus bellus* a careful study of the smear preparations reveals the fact that this second vacuole was formed through a diffusion of substance from the distal end of the central rod. This is clearly shown in the figures indicated. The second vacuole increases in size and ultimately surrounds completely the central body so that the spermatid presents the picture illustrated by Fig. 55. In this figure the primary and secondary vacuoles have changed into distinct vesicles and from now on they will be designated as the primary and secondary vesicles.

Soon a thin line grows out from the distal portion of the central body and this penetrates the upper end of the second vesicle to form a small opening (Fig. 56). The central body then begins to hollow out thereby becoming tubular. At the same time the outer opening of the second vesicle becomes collar-like in appearance. These changes are seen in Figs. 57–59. Following this the nucleus and mitochondrial ring fuse and become like one structure drawn up tightly around the vesicles (Fig. 60). From this last named nuclear-mitochondrial portion, the radial arms of the mature spermatozoa are formed (Fig. 61).

The last transformation in the mature spermatozoa occurs in the central body. The proximal end of this structure penetrates the tubular, distal end to form a small spine-like vertical rod (Figs. 62 and 63). This can only be seen in slides of spermatozoa which have been greatly destained. In other slides the spermatozoa all present the appearance of the one shown in Fig. 64. When such a male gamete that has been stained with Heidenhain's hæmatoxylin and acid-fuchsin is examined, the central body is uniformly black except for its distal end where it joins the dark collar-like opening, which is clear; the second vesicle is an amber color; the primary vesicle is colorless, and the nuclear-mitochondrial mass is darkly granular, with a darker ring immediately around the second vesicle. Such mature spermatozoa fill in the lower portion of the testis and the vas deferent ducts.

When these spermatozoa begin to open up one notices that four types of them exist, a three (Fig. 65), four (Fig. 66), five (Fig. 67) and six (Fig. 68) rayed type. The four and five rayed types predominate in largest numbers. The opening up and explosion of these spermatozoa has already been described in a previous contribution to this journal (Fasten, '21). As pointed out in that paper, the explosion is produced by a lowering in the osmotic pressure of the medium which surrounds the gametes.

SUMMARY.

1. During the latter part of June and early portion of July the testis of *Lophopanopeus bellus* (Stimpson) Rathbun is in the best condition for the study of the spermatogenesis process.

2. Primary and secondary spermatogonial divisions can be distinguished. The spermatogonial chromosomes are univalent and probably number around 124.

3. Large nutritive cells are frequently associated with spermatogonial strips in tubules where there are mature spermatozoa. These have irregular nuclei, and are, undoubtedly, produced from primary spermatogonia which have failed to mature.

4. The primary spermatocyte undergoes growth, parasynapsis, tetrad-formation and reduction division. There are 62 bivalent chromosomes seen in polar views of the metaphase stages of this division.

5. During the growth period a chromatoid body appears in the cytoplasm, and when the reduction division takes place this wanders undivided to one of the poles of the cell, resulting in the formation of two kinds of secondary spermatocytes, one which possesses the structure and the other which is devoid of it.

6. There is no rest period between the primary and secondary spermatocytes. The division of the latter is equational and produces two types of spermatids, one having a chromatoid body and the other which is minus such a structure. This last type is about three times as numerous as the former one.

7. At an early stage, the chromatoid body is expelled from the spermatids which contain it, and from then on all the spermatids undergo similar complicated transformations. These changes bring about the formation of the radial spermatozoa which are packed away in single spermatophores within the vas deferent ducts.

8. Four kinds of mature spermatozoa may be distinguished, namely, three-, four-, five- and six-rayed types. The four- and five-rayed spermatozoa are the ones which are encountered most frequently.

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EXPLANATION OF PLATES.

The figures in the accompanying plates were drawn with the aid of the camera-lucida at a magnification of 1,600 times. They are all from smear preparations.

Explanation of Plate I.

Fig. 1. Resting primary spermatogonium.

Fig. 2. Fragmentation of chromatin into smaller clumps, primary spermatogonium.

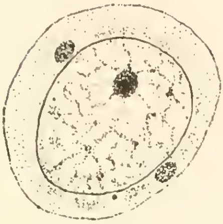
Figs. 3 to 7. Nutritive cells.

Figs. 8 to 11. Secondary spermatogonia in various stages of mitosis.

Fig. 12. Resting primary spermatocyte.

Figs. 13 and 14. Early growth stages, primary spermatocyte.

Fig. 15. Leptotene stage, primary spermatocyte.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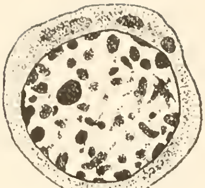
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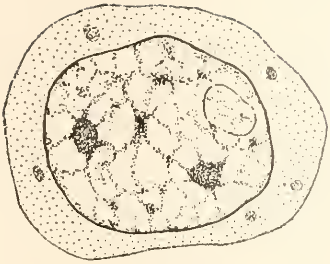
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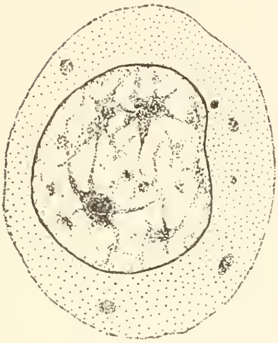
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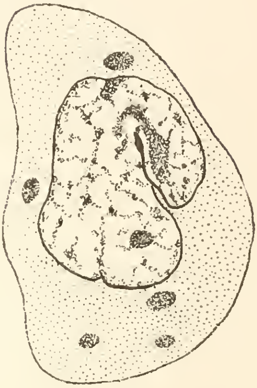
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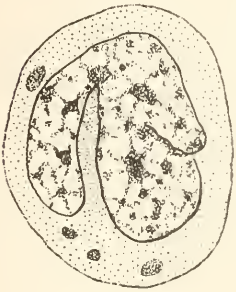
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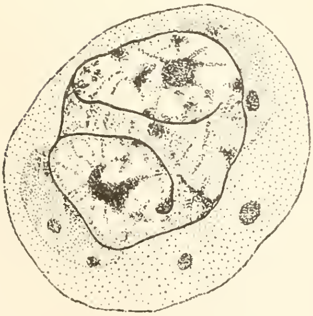
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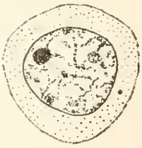
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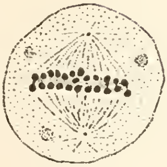
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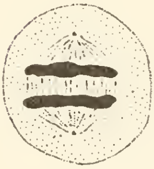
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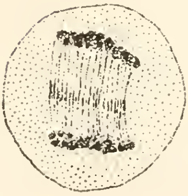
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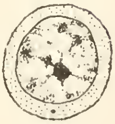
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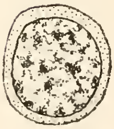
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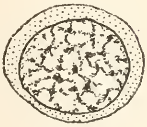
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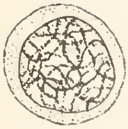
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Explanation of Plate II.

Fig. 16. Pre-synapsis stage, primary spermatocyte, showing the parallel, side-by-side arrangement of pairs of leptotene threads.

Fig. 17. Pachytene stage, primary spermatocyte.

Figs. 18 and 19. Diplotene and post-diplotene stages, primary spermatocyte.

Figs. 20 and 21. Pre-tetrad, tetrad and dumb-bell formation, primary spermatocyte.

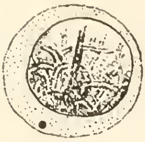
Figs. 22 and 23. Metaphase stages, primary spermatocyte.

Fig. 24. Polar view, metaphase, primary spermatocyte, showing sixty-two bivalent chromosomes.

Figs. 25 to 28. Stages in division of primary spermatocyte.

Figs. 29 to 34. Stages in division of secondary spermatocyte that possesses a chromatoid body.

Figs. 35 to 38. Stages in mitosis of secondary spermatocyte which lacks the chromatoid body.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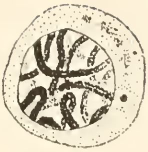
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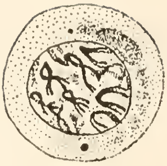
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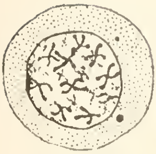
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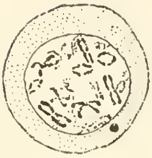
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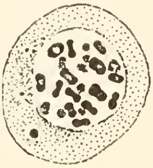
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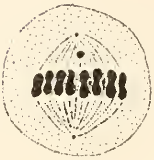
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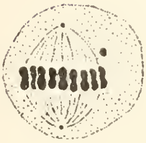
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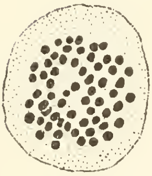
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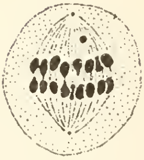
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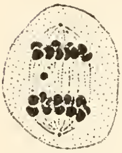
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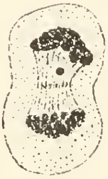
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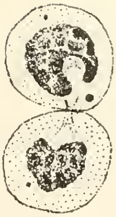
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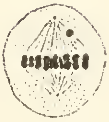
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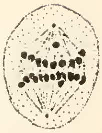
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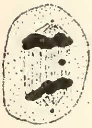
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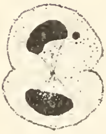
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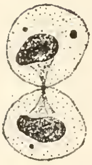
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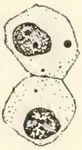
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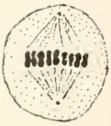
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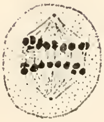
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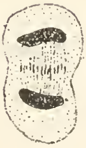
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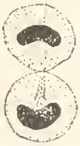
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NATHAN FASTEN.

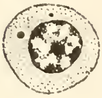
EXPLANATION OF PLATE III.

FIGS. 39 TO 43. Stages in transformation of the spermatid that contains a chromatoid body. In Fig. 43 the chromatoid body is expelled.

FIGS. 44 TO 47. Stages in the transformation of the spermatid that does not possess a chromatoid body.

FIGS. 48 TO 64. Later stages in transformation of both types of spermatids.

FIGS. 65 TO 68. The four types of spermatozoa produced.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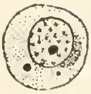
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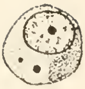
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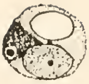
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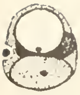
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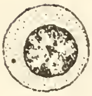
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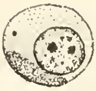
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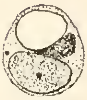
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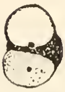
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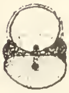
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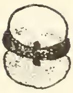
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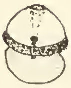
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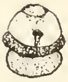
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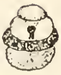
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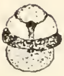
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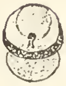
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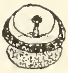
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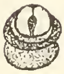
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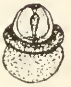
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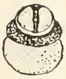
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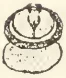
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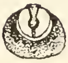
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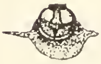
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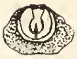
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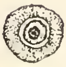
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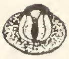
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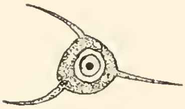
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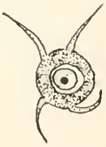
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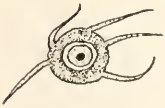
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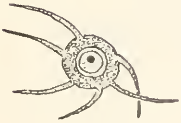
68

NATHAN FASTEN.

SOLE PATTERNS OF TWINS.

ROBERT B. MONTGOMERY, B.S., M.D.,

Instructor, Dept. of Clinical Med., Univ. of Wis.

In the study of twins, one frequently desires to know whether or not they are of monozygotic or dizygotic origin. This question can be answered by examination of the placenta and membranes at birth, but when older children and adults are studied it is difficult, and often impossible, to do so. Work has been done on the general physical appearance, mentality, palm patterns, whorls of the hair, etc., but no definite data has been secured. This study was undertaken in the hope that the friction-ridge patterns on the sole of the foot might reveal some clue as to the type of twin from whom they were secured.

Newman [1, 2] thinks that monozygotic twins are apt to have identical patterns, that they are found nearly always in this type of twin, but that unlike patterns do not disprove their monozygotic origin. Wilder [3] also has remarked upon the striking similarity which sometimes occurs. He [4] states that while they are similar grossly, the Galton details are always different. Accurate information on this subject is lacking.

Prints were secured from 87 pairs of twins living in Madison and vicinity; the process perfected by Professor Mathews being used in order that the fine details might be more readily studied. The youngest pair was one week old, and the oldest 85 years. Most of the subjects, however, were school children. Thirty-three pairs were male-male, 24 were female-female, and 30 were male-female. Of the 174 individuals examined, 96 were male and 78 female.

The ratio of monozygotic to dizygotic twins has been variously stated. Newman [1] thinks that the monozygotic type occurs about 25 per cent. of the time, while De Lee [5] quotes Ahlfeld as stating that 15.55 per cent. of all twins are monozygotic. Ahlfeld's figures are, perhaps, more reliable because he examined the placentas and membranes in 1,157 twin births.

The Wilder method is used in classifying the patterns and a summary of his method is as follows: The pattern bearing area on the ball of the foot is divided into two parts, one proximal to the great toe (hallucal) and the other proximal to the smaller toes (plantar). When three ridges meet to form a "Y" it is called a delta of which there are three in the hallucal area, one distal (just below the great toe), one medial, and one lateral. If all three of them are present the pattern is a whorl (W), if the distal one is absent the resulting pattern is a loop opening toward the great toe and is designated as an "A" pattern, if the medial one is absent and the loop opens medially a "B" pattern is formed, and if the lateral delta is not present a "C" pattern results. When no pattern is present it is called an open field (O). The plantar area contains three places in which a pattern may be found. These lie in the three interdigital spaces proximal to the four smaller toes. Four general types of patterns are found in these areas: the open field or no pattern, designated by an O, a loop opening distally (U), a loop opening proximally (∩), and a whorl (W). To illustrate the application let us turn to the right foot of twin 8 (Fig. 1). The hallucal pattern is a loop opening distally and is described as an 'A' pattern. The first plantar area also contains a loop opening distally but is designated by a 'U,' as is the second plantar area. In the third area there is no pattern (O). Bringing the various symbols together we have AUUO. To simplify matters, Wilder has proposed a table in which each combination of plantar patterns is given a number: O O O = 1, O O U = 2, O O ∩ = 3, etc. The combination here is 21, and the formula is thus abbreviated to A21. Proximal to the plantar areas there are frequently found other deltas which are designated by the symbol 'd,' and as there are two in this print, we arrive at the formula A21dd. To formulate the left foot we proceed in the same manner except that the plantar patterns are still read from left to right, giving the formula A6dd. The formula of the right foot is placed as the numerator of a fraction and the left foot as the denominator:

$$\frac{\text{A21dd}}{\text{A6dd}}.$$

In order that a set might be called identical the following conditions must be met: (1) either all four patterns, or both right and both left patterns must have the same formula, and (2) there must be no marked differences in the patterns even if they have the same formula. As space does not permit a more detailed description of the Wilder method of classification, the reader is referred to his writings on the subject. Fig. 1 illustrates a set of identical patterns. On close examination the similarity of the patterns of each individual is striking. In this series 13 sets (14.94 per cent.) are identical (Table II.), and all except

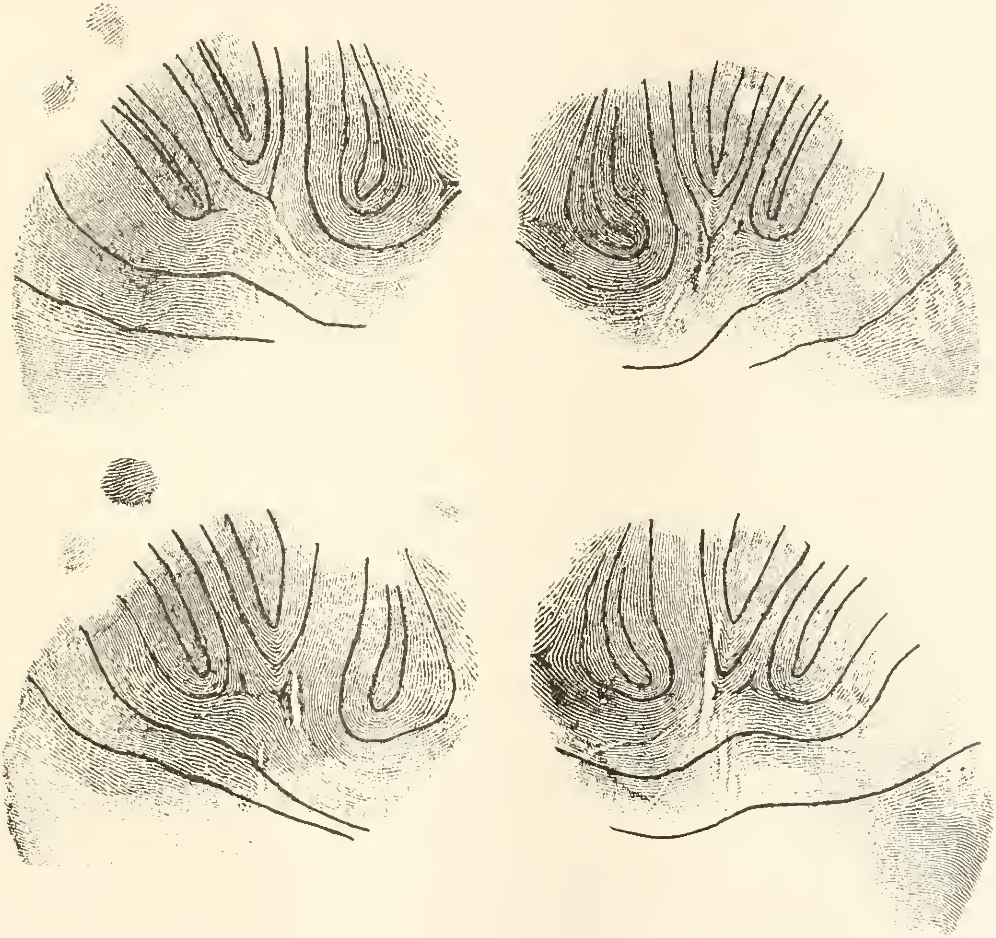

FIG. 1. Twins 7–8. Formula: $\frac{\text{A21dd}}{\text{A6dd}}$. Some of the lines have been inked in order that the patterns might be more readily seen.

one occurred in like-sex twins. The striking points of similarity in the different sets are enumerated below. Table I. gives the complete list of patterns. Although twins 25–26 have the same formula, they are not classed as identical because of the very rudimentary character of one of the patterns and so do not meet the conditions stated above.

TABLE I.

COMPLETE LIST OF ALL TWIN PATTERNS.[1]

No.	Sex.	Right.	Left.	No.	Sex.	Right.	Left.	No.	Sex.	Right.	Left.
1	♀	W38dd	W23dd	59	♀	A5d	A5d	117	♀	W5d	B5d
2	♂	A1	A1	60	♀	A5d	A21dd	118	♀	W7d	W1
3	♂	A37d	A5d	61	♂	A5d	A5d	119	♂	A6dd	A21dd
4	♂	A5d	A1	62	♂	A1	A5d	120	♂	A6dd	A21dd
5	♂	W25d	B5d	63	♀	A17d	A1	121	♀	A1	A1
6	♂	B5d	B5d	64	♂	W13d	W13d	122	♂	A1	W3
7	♀	A21dd	A6dd	65	♂	A5d	A1	123	♂	A6dd	A21dd
8	♀	A21dd	A6dd	66	♂	A5d	A5d	124	♂	A6dd	A5d
9	♀	W14ddd	W13d	67	♂	A5d	A5d	125	♂	A5d	A5d
10	♀	W12	W5d	68	♂	A5d	A1	126	♀	A6dd	A1
11	♂	B5d	A5d	69	♂	A1	A1	127	♀	A5d	A11
12	♀	A1	A1	70	♀	A37d	A7d	128	♀	W5d	A5d
13	♀	A29dd	A6dd	71	♀	A37d	A7d	129	♂	O45d	O15
14	♀	A5d	A5d	72	♂	A57d	A1	130	♀	A13d	W15d
15	♂	W5d	W5d	73	♀	A6dd	W5d	131	♂	O1	A1
16	♂	W5d	W5d	74	♀	W5d	W5d	132	♀	A5d	B1
17	♂	A5d	A5d	75	♀	B1	B1	133	♂	A1	A1
18	♂	A37d	A7d	76	♀	B1	A1	134	♀	A1	A1
19	♀	W5d	W5d	77	♀	B45d	W7d	135	♀	W5d	W5d
20	♀	W5d	W5d	78	♀	B45d	W7d	136	♀	A1	A1
21	♀	A6dd	A1	79	♀	W1	W1	137	♀	A1	A1
22	♂	W33	W1	80	♀	A5d	A3	138	♀	B5d	A5d
23	♂	A37d	A5d	81	♀	W6dd	W5d	139	♂	A5d	A7d
24	♂	A5d	A5d	82	♀	A5d	W5d	140	♂	W5d	W5d
25	♂	W5d	W5d	83	♂	W49d	W4d	141	♂	W5d	W5d
26	♀	W5d	W5d	84	♂	W61dd	W12d	142	♀	A2d	A7d
27	♀	A5d	A5d	85	♂	A5d	A5d	143	♀	W1	W3
28	♂	W45d	W15d	86	♂	A5d	A5d	144	♀	A37d	W1
29	♂	A5d	A1	87	♀	A45d	A9	145	♂	B1	B1
30	♂	A5d	A1	88	♂	A45d	W13d	146	♂	A33	A1

TABLE I.—(*Continued.*)

No.	Sex.	Right.	Left.	No.	Sex.	Right.	Left.	No.	Sex.	Right.	Left.
31	♀	A5d	A5d	89	♀	A5d	A5d	147	♀	A2d	A5d
32	♂	B14dd	B29dd	90	♀	W1	W5d	148	♂	A37d	A5d
33	♂	W5d	W5d	91	♂	A5d	A5d	149	♂	W13d	A43
34	♀	A37d	A3	92	♂	A52dd	A6dd	150	♂	W41d	W15d
35	♂	A1	A1	93	♂	W2d	W21dd	151	♂	A5d	A5d
36	♂	A1	A1	94	♀	B6dd	B21dd	152	♂	A5d	A5d
37	♂	A1	A1	95	♂	A5d	A1	153	♂	A5d	A5d
38	♀	A5d	A1	96	♀	B6dd	B5d	154	♂	A6dd	W5d
39	♀	B38dd	B7d	97	♀	A1	W1	155	♀	O38d	O5
40	♂	W37d	W7d	98	♂	A5d	A5d	156	♀	O46d	A15d
41	♂	A6dd	A21dd	99	♀	A33	W9	157	♀	A17d	B1
42	♂	A5d	A7d	100	♀	W45d	W11	158	♂	W5d	W5d
43	♂	W46dd	W7d	101	♂	A1	W1	159	♀	W1	W1
44	♂	A45d	W15d	102	♂	A5d	W5d	160	♀	A5d	A5d
45	♂	A5d	W1	103	♂	W1	W1	161	♂	A33	A2d
46	♂	A1	A1	104	♂	W1	W1	162	♂	W5d	B5d
47	♂	A2d	A17d	105	♂	W5d	W5d	163	♂	W5d	W5d
48	♂	A5d	A5d	106	♂	B1	B5d	164	♂	B6dd	B23dd
49	♀	W5d	A5d	107	♂	A5d	O5	165	♂	A1	W1
50	♀	A5d	A5d	108	♂	W37d	W7d	166	♂	A5d	A5d
51	♂	A1	A1	109	♀	A5d	A1	167	♀	A38dd	A1
52	♀	A17d	A2d	110	♀	A5d	A1	168	♀	A6dd	A21dd
53	♂	W37d	W47d	111	♀	O15d	O29d	169	♀	A41	A11
54	♀	A5d	A21dd	112	♂	W57d	W3	170	♀	A41	A11
55	♂	A5d	W5d	113	♀	A1	A5d	171	♂	A37d	A3
56	♀	W37d	W7d	114	♀	A1	W1	172	♂	A1	A5d
57	♂	A5d	W7d	115	♂	W13d	W5d	173	♂	A38dd	A63dd
58	♀	W13d	W47d	116	♂	W37d	W7d	174	♀	A5d	A7d

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[1] In this table ♂ means male, and ♀ means female. The twins are grouped in pairs.

Twins 7–8.—Note the odd shape of the hallucal pattern "A" in both right feet and the pointed character of the first plantar pattern in all four feet. The plantar patterns of the "U" type are usually rounded. The ridge counts from the cores of the hallucal patterns to the cores of the first plantar patterns are: 7R, 37; 7L, 38; 8R, 38; 8L, 39. From the first to the second plantar patterns the count is: 7R, 28; 7L, 30; 8R, 27; 8L, 29.

TABLE II.

THIS GIVES ONLY THOSE PATTERNS WHICH ARE IDENTICAL.[2]

No.	Sex.	Right.	Left.	No.	Sex.	Right.	Left.
7	♀	A21dd	A6dd	103	♂	W1	W1
8	♀	A21dd	A6dd	104	♂	W1	W1
15	♂	W5d	W5d	109	♀	A5d	A1
16	♂	W5d	W5d	110	♀	A5d	A1
19	♀	W5d	W5d	119	♂	A6dd	A21dd
20	♀	W5d	W5d	120	♂	A6dd	A21dd
29	♂	A5d	A1	133	♂	A1	A1
30	♂	A5d	A1	134	♀	A1	A1
35	♂	A1	A1	151	♂	A5d	A5d
36	♂	A1	A1	152	♂	A5d	A5d
77	♀	B45d	W7d	169	♀	A41	A11
78	♀	B45d	W7d	170	♀	A41	A11
85	♂	A5d	A5d				
86	♂	A5d	A5d				

Twins 15–16.—Although this is a common pattern, all hallucal patterns are counter-clockwise, and all except one are of the lateral pocket variety.

Twins 19–20.—Here, too, all hallucal patterns are counter-clockwise. The ridge counts from the hallucal to the second plantar patterns are: 19R, 73; 19L, 72; 20R, 83; 20L, 80.

Twins 29–30.—This is a common pattern. The prints are too blurred to do a ridge count accurately.

Twins 35–36.—All show finer lines in the plantar areas although there are no patterns present.

Twins 77–78.—Both right feet and both left feet have the same pattern. The plantar areas are of a type not usually seen.

Twins 85–86.—There are suggestions of patterns in the third plantar areas on all four feet.

Twins 103–104.—All hallucal patterns are of the seam variety.

Twins 109–110.—Both right feet and both left feet have the same pattern. The left feet (and not the right) have fine lines in the plantar areas although there are no patterns present.

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[2] In this table ♂ means male, and ♀ means female. The twins are grouped in pairs.

Twins 119–120.—The general type of patterns found are not a very common variety. The ridge counts from the second to the third plantar areas are: 119R, 39; 119L, 37; 120R, 27; 120L, 25.

Twins 133–134.—In this set the twins are of opposite sex. This type of pattern is of common occurrence.

Twins 151–152.—The plantar patterns on both left feet have a "V" shape.

Twins 169–170.—This type of pattern is uncommon.

An attempt was made to count the ridges from the various landmarks, but they vary so greatly in most instances that it was not thought worth while to report all of the figures. The striking similarities are given above.

The individual patterns occur in almost the same ratio as those found in a previous series.[6]

The fact that twins 133–134 are of opposite sex might lead one to conclude that study of the sole prints is without value in determining the type of twin under consideration, but I feel that such is not the case. They are of the A1 type, one of the simplest and commonest. I have found that A1 patterns occur about 10 per cent. of the time on both feet, and so do not think it unreasonable to assume that their presence in this set of twins is an accident.

In order to determine whether or not patterns of the same formula might be found on all four feet of ordinary (not twin) brothers and sisters, the prints of 38 pairs (the only ones available at this time) were examined. Of these 10 were female-female, 12 male-male, and 16 female-male. One pair of sisters had the same patterns, but they were of a very common type, A5d.

Conclusions.

A study of the above data leads one to the conclusion that the presence of identical patterns on the soles of a pair of twins might point to their monozygotic origin, but as Newman states, their absence does not disprove it. A study such as this is, of course, not conclusive, but it points the way for further investigation. It was undertaken in the hope that sole patterns might reveal some information as to the origin of twins. If the placentas and patterns of a large series could be examined at birth, the question would be answered.

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SPERM FILTRATES AND DIALYZATES.

Their Action on Ova of the Same Species.[1]

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Introduction.

The idea persists that spermatozoa bear a specific substance which is essential in fertilization. Yet all attempts to extract from spermatozoa a substance which will cause development of ova of the same species have failed or have been open to grave criticism. It is now possible to avoid certain errors which invalidated the results of these investigations. The experiments, to be reported here, were undertaken to determine whether, by processes of filtration and dialysis, solutions can be obtained from living sperm which will effect activation and development of ova of the same species. For interesting me in this problem and for invaluable aid in its solution I am indebted to Dr. Otto C. Glaser.

The experimental work was carried on during the summers of 1919–1922 at the Marine Biological Laboratory at Woods Hole, Massachusetts, and from December until June 1920–1921 at the

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Hopkins Marine Laboratory at Pacific Grove, California. I wish to express my appreciation of the hospitality extended to me at these laboratories, and my thanks to their Directors, Dr. Frank R. Lillie and Dr. Walter K. Fisher, for their aid and encouragement.

The fact that the spermatozoa, rather than the medium which carries them, are the essential agents in fertilization was established in 1824 by Prevost and Dumas; and it is now conceded that fertilization is monospermic and involves the combination of nuclear material from a single spermatozoön and a single ovum. In many species only a single spermatozoön penetrates the cytoplasm of the egg, as was shown by Hertwig (1876); but even when in normal fertilization more than one enters the egg, as in the pigeon, the pro-nucleus of only one enters into the formation of the fertilization nucleus (Harper, 1904, and Blount, 1909). As stated by Glaser (1915), "a single spermatozoön is sufficient to carry on the biparental effect."

It is well known, however, that in the process of fertilization in any species the spermatozoa far exceed the ova in number. This was first noted in frogs by Spallanzani (1785), and verified by Prevost and Dumas (1824). Under such conditions direct contact of every ovum with a single spermatozoön is possible. But mere contact does not ensure fertilization. Even in physiological solutions spermatozoa rapidly lose their fertilizing power, Vernon (1899); Gemmil (1900); Dungay (1913); Lillie (1915) and Cohn (1918). Two explanations have been offered in recent years to account for the excess of spermatozoa. Lillie (1915) implies that in an excess at least one spermatozoön *possessing sufficient fertilizing power* ("sperm receptors") will reach each ovum. On the other hand Glaser (1915) and Cohn (1918) state that the extra spermatozoa may produce changes in an ovum which facilitate the inclusion of a single spermatozoön.

The idea of a superficial effect of sperm on ova is supported by results obtained with *Nereis limbata*, Lillie (1911) and Goodrich (1920); with *Arbacia punctulata*, Lillie (1921); with *Sabellaria alveolata* and *Halosydna gelatinosa*, Labbe (1921, 1922); and with *Asterias*, Chambers (1923). There are indications that a chemical substance or substances are produced by the sperm:

Godlewski (1911); Herlant (1912); Heilbrun (1915); and Lillie (1911, 1915, 1921).

What real evidence have we to support the suggestion, recurrent in one form or another, that the sperm exude a substance which facilitates fertilization? The early attempts of Spallanzani (*loc. cit.*), with filtrates, and of Prevost and Dumas (*loc. cit.*), with filtrates and extracts of spermatozoa, to demonstrate such a substance gave purely negative results. In later work: Pierri (1899); Winkler (1900); Du Bois (1900); Gies (1901); Pizon (1905); Jacoby (1910); Morse (1912); Robertson (1912); Foa (1918)—positive results, where reported, are actually vitiated by errors of technique or interpretation, Loeb (1913, p. 104, and 1916, p. 203); and Lillie (1919, pp. 133, 134).

II. METHODS.

In an investigation of this kind so much depends upon the methods employed that it seems desirable to present these in some detail.

All glassware was cleaned with potassium-bichromate-sulphuric acid mixture, subsequently removed by washing in fresh and in sea-water. All solutions were made with analyzed chemicals (Kahlbaum's, Merck's, and Baker's) with water redistilled from glass. Specific gravity was determined in early experiments with a standard hydrometer and later with a Westphal balance. A special set of indicators and standards provided and tested at intervals by Hynson, Westcott and Dunning was used for the colorimetric determination of the hydrogen-ion concentration, Clowes and Smith (1923 and 1924). Every possible precaution was taken to avoid accidental insemination and sea-water controls accompanied every experiment.

Method of Obtaining Ripe Reproductive Cells of Sea-urchins.—One method is as follows: The instruments and hands of the operator are thoroughly washed in tap water and the animals are washed in tap water followed by sea-water. They are opened by cutting through the oral disc with sterile scissors, the disc and the alimentary canal are removed with sterile forceps and the cœlom is flushed with filtered sea-water to remove the body fluids and any intestinal contents. Males and females are placed on opposite sides of the operator. Each animal, unless shedding freely, is immediately wiped and placed on its aboral surface in a Syracuse watch glass. In this position the reproductive cells exude through the genital pores. In a second method suggested by Dr. Glaser, the spines are rubbed off before the animals are washed and dried. If the reproductive elements are ripe, shedding begins immediately and it is unnecessary to cut the "test." There is no admixture with body fluids or with sea-water and very clean dry reproductive cells are obtained. Only ripe spermatozoa are shed and these are collected in a beaker. Not all the eggs shed are ripe. For this reason it is necessary to fertilize a sample of eggs from each female. After this the "certified" eggs are placed together in a finger bowl with sufficient sea-water to keep the concentration of egg-water below a point at which it will injure the eggs.

Method of Obtaining Ripe Eggs of Nereis limbata.—The method recommended by Lillie (1911) and Just (1915*a*) was employed. Males and females as they were caught were segregated in finger bowls of sea-water and kept cool until all preparations for an experiment were complete. The most satisfactory results are usually obtained with reproductive cells taken from such animals within two hours after collection; although, if necessary, shedding can be prevented for twelve hours or more if the dishes containing the animals are kept on ice. Since either drying the animals on filter paper or washing them in distilled water leads to shedding, each animal was "sterilized" by first transferring it to a finger bowl containing two hundred and fifty milliliters of sea-water. (In this volume of sea-water any sperm adhering to the body of the female will lose their fertilizing power.) Subsequently each animal was placed in a dry Syracuse watch glass in which any excess of moisture was absorbed with strips of filter paper, and there cut transversely with sterilized scissors. The eggs or sperm were forced out by the spasmodic contractions of the body muscles. To avoid accidental insemination the males were opened with a second set of instruments; and all inseminated controls were kept separate from the experiments in which special solutions were being tested. Eggs and sperm were kept covered and cool until used.

Preparation of Filters.—Berkefeld and Mandler diatomaceous filters were used: in preliminary experiments the coarse and medium grade of Berkefeld filters; and in all other work Mandler filters, tested to six to twelve pounds air pressure in the size two and one half by five eighths inches. The latter are used generally in bacteriological work. These were boiled in five per cent. aqueous solutions of sodium bicarbonate, washed and boiled repeatedly in redistilled water until the wash water was neutral in reaction. Finally they were thoroughly washed in filtered sea-water. After this a stream of sea-water was passed through the filters until samples of such water produced no injurious effect on unfertilized ova and no disturbance of fertilization or of development. Such filters were considered "clean." Every filter was subjected to this treatment each time that it was used. The necessity for these precautions was indicated by a variety of experiences. The first sea-water passed through a boiled and washed filter may be sufficiently hypotonic to cause cytolysis of ova within twenty-four hours. Unless all alkali is removed the filtered sea-water may produce activation of ova. The use of acid in the cleaning of filters is prohibitive because the filtrates would then contain traces of heavy metal dissolved from the filter bands. The impurities in question are often too slight for detection by chemical tests, but are only too clearly revealed by their effects upon unfertilized or fertilized ova.

Preparation of Sperm Filtrates.—Five and ten per cent. suspensions of spermatozoa were prepared by adding to a definite volume of dry sperm a measured quantity of sea-water. These were allowed to stand at room temperature (fifteen degrees centigrade at Pacific Grove and twenty-two degrees centigrade at Woods Hole) for from five minutes to four hours. To ensure the greatest activity of the spermatozoa the carbon dioxide generated by them was prevented from accumulating by a thorough aëration of the suspensions. The latter were then either clarified first by centrifuging or by filtering through filter paper, or were transferred directly to a diatomaceous filter. Contamination of the filtrates by back suction was prevented by collecting the filtrates into Pyrex test tubes set in the suction flasks.

During the passage of the filtrate it is necessary to prevent or at least to minimize the destruction of spermatozoa by dehydration or compression. This was attempted by not allowing the surface of the mantle to become exposed to air and by frequent and cautious stirring of the suspension. Some of the spermatozoa are not seriously injured in the process of filtration as indicated by the isoagglutinable and fertilizing power of the sperm remaining on the surface of the mantle at the end of the process.

The filtrates, which are automatically freed from any excess of carbon dioxide in passing through the filter mantle, are then transferred to flasks which are kept tightly plugged and placed in a refrigerator. These solutions remain free from bacteria and retain their peculiar physiological properties for at least a month.

Preparation of Sperm Dialyzates.—There is one objection to the use of sperm filtrates. Some spermatozoa may undergo destruction on the surface of the mantle. To meet this objection I prepared dialyzates of sperm in order to compare their action on ova with that of filtrates.

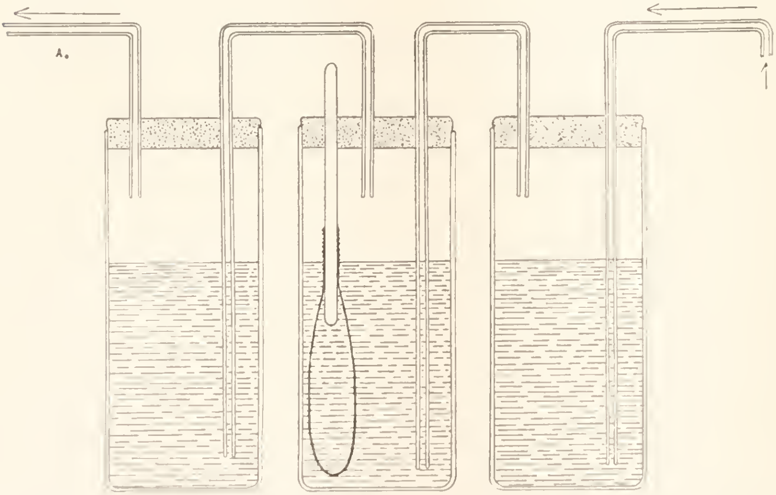

FIG. 1. Dialyzing apparatus. Air is drawn through the sea-water in the three bottles by suction exerted at *A*. The collodion sac is suspended in the sea-water in the central bottle and this sea-water dialyzate used in experiments later. The outer two bottles serve as safety flasks.

Collodion sacs used as dialyzers were made in fifty milliliter centrifuge tubes from a twelve per cent. collodion prepared according to the method of Gates (1921). The sacs were washed in redistilled water and in sea-water. Samples of sea-water, allowed to stand in the washed sacs for at least twelve hours, were tested in the same manner as that passed through the diatomaceous filters. Tests for leakage were made and any imperfect sacs were discarded. Ten milliliters of twenty-five or of fifty per cent. suspensions of sperm were placed in a tested collodion sac and dialyzed against forty milliliters of sea-water. The resultant dialyzates may be considered comparable to filtrates prepared from five and ten per cent. suspensions of sperm. The sea-water dialyzates must be aërated constantly to provide the most favorable conditions for the sperm during the process. This involves two dangers: evaporation and contamination of the dialyzates. In order to eliminate these sources of error the method illustrated in Fig. 1 was employed.

In these experiments dialysis was continued for five to twenty-four hours. With one exception the sperm in the collodion sacs retained their fertilizing power and their capacity for iso-agglutination at the end of the process.

III. Properties of Preparations.

a. Physical and Chemical Properties.—The osmotic pressure of filtrates and dialyzates, as indicated by the specific gravity tests, is the same as that of sea-water. There is a slight variation in some preparations and in sea-water but it is not sufficient in itself, as determined by experimentation, to produce activation. The hydrogen-ion concentration of filtrates and dialyzates was also equal to that of sea-water (p_H 7.9–8.1 at Woods Hole; 7.6–7.8 at Pacific Grove), indicating that the carbon dioxide formed by active sperm was completely removed by aëration and that the sperm added no other free hydrogen-ions. There is a possibility that small amounts of acid may have combined with buffers in sea-water. The question now arises whether these preparations contain any active physiological principle. No living spermatozoa or fragments were ever observed, nor was there ever a case of normal fertilization in any of the preparations. It is also certain that they contain no chemical substance whose concentration falls within the range of sensitivity of the usual chemical methods.

Experiments in which blood plasma was passed through a Berkefeld filter, Cramer and Pringle (1913), and Goddard (1914), demonstrated that the filter held back for a time, not only suspended elements, but the various proteins in colloidal solution, but the third portion passed through the filter contained some fibrinogen. Mudd (1922) suggested that in alkaline solution protein may be carried through in small amounts. The filter itself is negatively charged and because of the amphoteric properties of protein the latter dissociates as an anion in alkaline solutions and so would tend to be carried through the filter.

It is important therefore to determine if possible whether nitrogen compounds are present since Loeb (1914) states that protamine will induce the first cell divisions in eggs of *Arbacia;* and Labbe (1923) using a one to forty thousand solution of sodium nucleinate in sea-water on unfertilized eggs of *Arbacia* obtained some swimming larvæ. Because of buffer action these solutions had the same hydrogen-ion concentration as sea-water, and Labbe concluded that the sodium nucleinate exerted a specific action not due to its hydrogen-ion concentration.

All the usual tests for protein or protein split products such as guanine, protamine, and nucleic acid were negative. A micro-kjehldahl test, performed for me by Dr. W. Dennis, was also negative, indicating that nitrogen is not present in sufficient amounts to be detected by this method. Incineration tests gave evidence of the presence of carbon in larger amounts in both filtrates and dialyzates than in sea-water.

b. Physiological Properties.—From the presence of carbon in these preparations it is certain that they contain something of organic origin. It seemed possible that they might produce changes in the ova of foreign species, since sperm extracts obtained by other investigators have produced marked effects on ova of foreign species even when they had no influence on ova of the same species, Loeb (1916, p. 102). Consequently tests were made to determine whether *Arbacia* sperm filtrates would affect the ova of *Nereis limbata*, a form in which maturation follows insemination. Seventeen experiments were performed in which ten preparations were used on *Nereis* eggs. The results obtained resemble in many respects those produced by insemination with *Nereis* sperm. The foreign filtrate causes production of jelly, the formation of a "fertilization membrane," complete maturation, and segmentation leading to the development of larvæ.[2] The latter however are abnormal in shape and in the distribution of cilia. In no case did these changes occur in sea-water controls. A protocol of one experiment is given below.

TABLE I.

CHANGES IN THE OVA OF *Nereis* EXPOSED FOR TWENTY MINUTES TO A FILTRATE PREPARED FROM A TWO PER CENT. SPERM SUSPENSION OF *Arbacia*.

Date.	Exper.	Per Cent. of Eggs Forming Jelly and Membranes.	Per Cent. of Dividing Eggs.	Per Cent. Swimming.
8/ 4/20	31	100	10	0.5
8/ 5/20	33	99	90	0
8/ 7/20	35	81	75	5
8/ 8/20	37	85	85	9
8/ 8/20	38	100	14.5	2
8/13/20	40	100	1	0
8/14/20	43	100	5	2
8/14/20	44	100	13.5	1
8/14/20	45	100	12	0.5
7/27/21	225	33	31.5	1
7/27/21	225	30	28.5	1

In experiments 31, 40, and 43 the ova were stale. In all cases membrane formation and maturation occurred as rapidly in ova in sperm filtrates as in inseminated ova. Subsequent development in ova treated with filtrate was delayed.

The *Arbacia* sperm filtrate acted like a parthenogenetic agent, yielding results similar to those obtained in *Nereis* with other methods (Lillie, 1911, and Just, 1915).[2] I obtained similar results with filtered egg-water of *Arbacia*. The latter observations, quoted by Dr. Alvalyn Woodward (1921), were verified frequently in subsequent experiments. Further experimentation demonstrated that the effect produced by sperm filtrates on *Nereis* eggs bears a definite relation to the strength of the preparations and to the duration of exposure of ova to them. The main question however is what effect have such filtrates and dialyzates on ova of the same species.

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[2] The developing ova divided into two, four and eight cells, and subsequently some of them developed into abnormal ciliated trochophores. The rest cytolyzed.

IV. The Potency of Sperm Filtrates and Dialyzates on Ova of the Same Species.

a. Arbacia punctulata.—The procedure employed to determine the effect of these preparations on ova of the same species and the results obtained are described first for *Arbacia*. The ova were exposed to the action of sperm filtrates and dialyzates in the proportion of two milliliters of fresh washed eggs to twenty-five milliliters of the test solution. Sea-water controls accompanied every experiment and samples of eggs from test solution and from sea-water were examined at intervals.

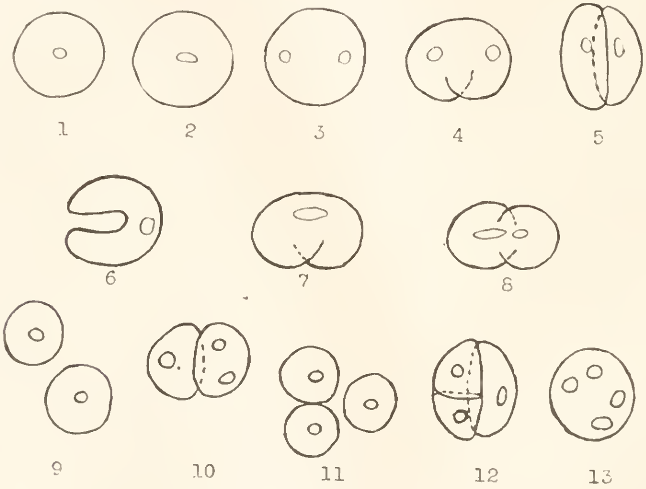
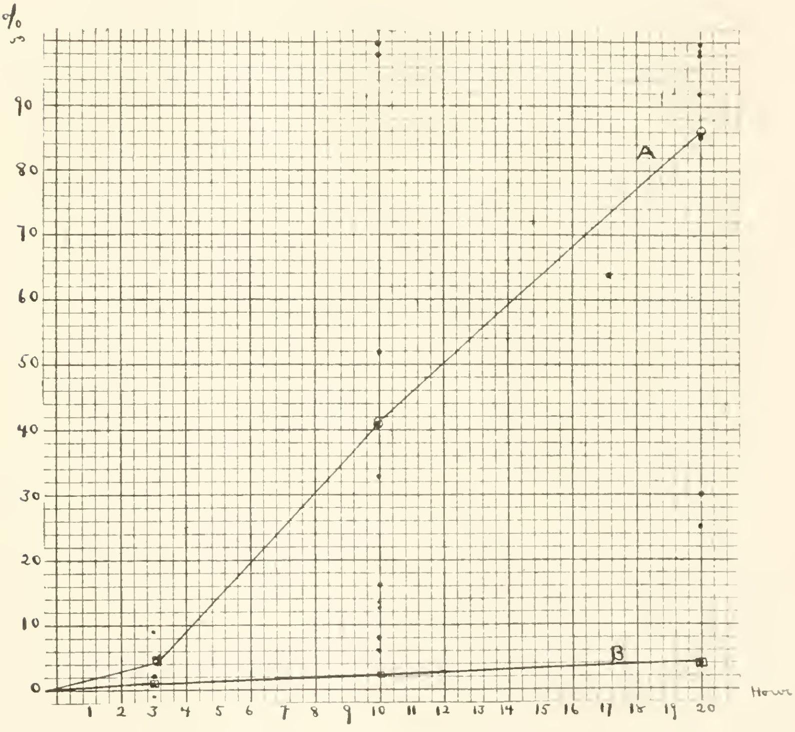


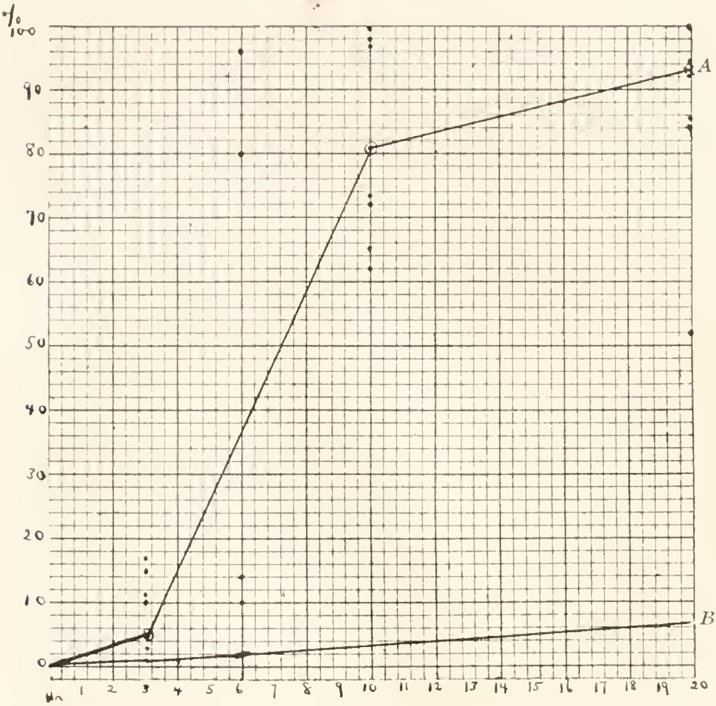
Fig. 2. Changes in shape and nuclear changes in ova exposed to sperm filtrates. 1–5 = changes in a single ovum within forty-five minutes; 6–13 = ova representing various changes produced by filtrates. Magnification × 350.

The first evidence of the influence of the filtrate or of the dialyzate on the ova is a change in the density of the cytoplasm at or near the center of the ovum, similar to that which precedes nuclear division in inseminated eggs. Indeed nuclear changes and nuclear division follow. Simultaneously a distortion of the egg occurs followed by its partial or complete cleavage into two or more parts. The cleavages are at times perfectly regular, at times unequal. Even the smallest cells are usually nucleated. Segmentation sometimes proceeds to the eight-celled stage, but in any case is followed, if the eggs remain in the filtrate, by a separation of the parts and their ultimate cytolysis. In a very few cases a slightly elevated membrane is visible. These results were reproduced with different lots of eggs and with different filtrates and dialyzates. In no case did these changes occur in sea-water controls or in sea-water filtered or dialyzed. The latter results indicate that no substance is given off by the filters or collodion sacs that can account for the effects of sperm filtrates and dialyzates.



Graph I. The percentage of ova divided and cytolyzed by sperm filtrates of the same species—*Arbacia punctulata.* *A* = the average of ten experiments with filtrates made from 2 per cent. suspensions. *B* = the average of ten sea-water controls. ● = percentages in individual experiments with sperm filtrates.

Confirmation of these results was obtained in a series of forty-two experiments with filtrates and twenty-four experiments with dialyzates. Within twenty-four hours the majority of ova in the test solutions had undergone decided changes, while those in controls remained normal in appearance. This is well illustrated in the percentages indicated in graphs I., II., and III.[3]



Graph II. The percentage of ova divided and cytolyzed by sperm filtrates of the same species—*Arbacia*. *A* = the average of ten experiments with filtrates made from 5 per cent. suspensions. *B* = the average of ten sea-water controls. ● = percentages in filtrates in individual experiments.

It is evident that some ova, probably because of a slight difference in physiological condition, are more rapidly affected by the filtrates and dialyzates than others, thus indicating a varying degree of susceptibility of the eggs to the action of the preparations.

In view of the failure of other investigators of this problem it is important to emphasize the fact that I obtained consistent results. At the same season of the year the eggs of different females are about equally susceptible, and filtrates or dialyzates prepared in the same manner are equally effective on ova of the same individual, as indicated in graphs IV. and V.

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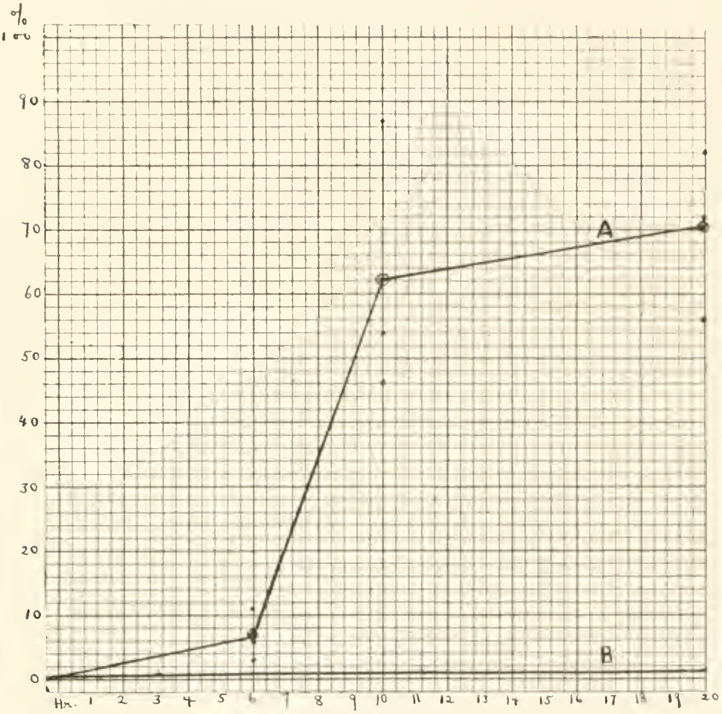
[3] The graphs indicate the number divided and cytolyzed rather than simply the ones in a state of division. Since the ova do not divide simultaneously, some have divided and cytolyzed at a time when others are undergoing nuclear changes or are dividing.

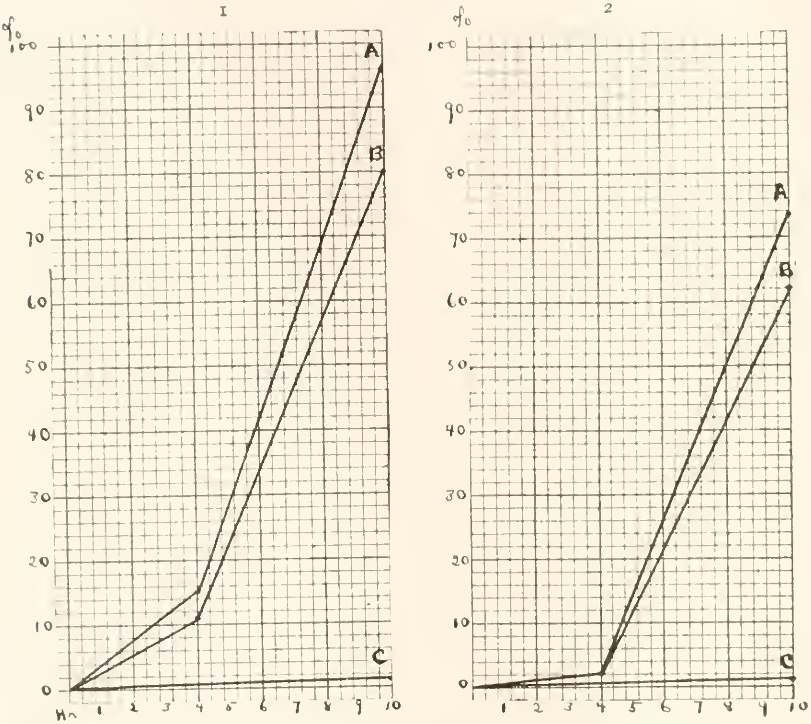


Graph III. The percentage of ova divided and cytolyzed in sperm dialyzates of the same species—*Arbacia punctulata*. *A* = the average of three experiments with dialyzates from 50 per cent. suspensions. Duration of dialysis = ten hours. *B* = the average of three experiments with sea-water dialyzed for ten hours against sea-water. ● = percentage in dialyzates in individual experiments.

It is apparent that, if the eggs are in the same physiological condition and are exposed at the same temperature to filtrates of equal strength, one may expect equal amounts of segmentation and cytolysis in approximately equal lengths of time.

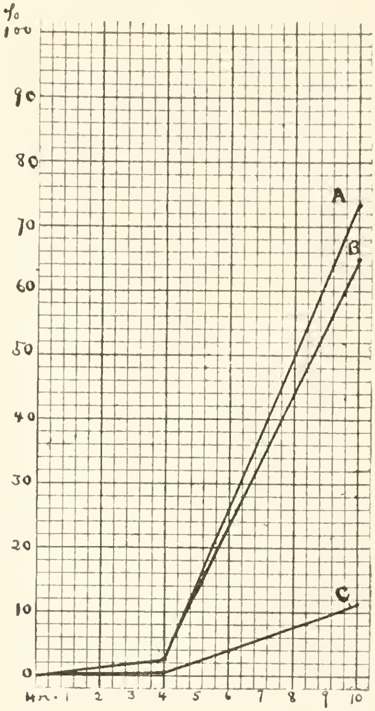
As might be expected a definite correlation exists between the strength of the test solutions and the percentage of eggs affected in a given time. This is well illustrated in graphs VI., VII., VIII., and IX.

The definite correlation which exists between the duration of exposure and the percentage of eggs affected suggested experiments in which the eggs are removed from the filtrate before the latter have had opportunity to produce any visible changes in the eggs. If now there are invisible effects and if these are orderly and significant, they should become noticeable after the eggs are transferred to sea-water. Cases in point are illustrated in graphs X., XI., and XII., and indicate clearly that the gross visible effects, distinct only after several hours, are preceded by important changes which may develop in some of the ova within a very few minutes. These changes may, after transfer of the ova to sea-water, lead to progressive changes in the ova.



Graph IV. Percentage of division and cytolysis of ova produced by sperm filtrates on two sets of eggs—*Arbacia punctulata*. 1 = Filtrate 6/30/21 from a 5 per cent. suspension. 2 = Filtrate 7/2/21 from a 5 per cent. suspension. *A* = one set of eggs. *B* = second set of eggs. *C* = control eggs in S.W.

The Effect of Filtrates Prepared from Heated Sperm.—The statement was made by Winkler (1900) and by Morse (1912) that when sperm suspensions were heated to 50°–60° centigrade the solutions failed to affect eggs. I therefore made filtrates from suspensions of *Arbacia* sperm heated to 42°–50° centigrade to compare their action with those of filtrates from unheated



Graph V. Percentage of ova divided and cytolyzed by two similar filtrates of sperm of the same species—*Arbacia*. *A* = Sperm filtrate 6/30/21 from a 5 per cent. suspension. *B* = Sperm filtrate 7/2/21 from a 5 per cent. suspension. *C* = Sea-water control.

sperm suspensions. The spermatozoa lose their iso-agglutinable and their fertilizing properties at a temperature of 38°–40° centigrade and tend to adhere to one another. One heated suspension was first passed through filter paper and then through a Mandler filter. As a control a part of the same suspension, unheated, was similarly treated. A comparison of the effect of the two filtrates is given in the following table.

Table II.

A Comparison of the Action of Filtrates Prepared from Heated and from Unheated Sperm Suspensions of *Arbacia* First Passed through Whatman Filter Paper.

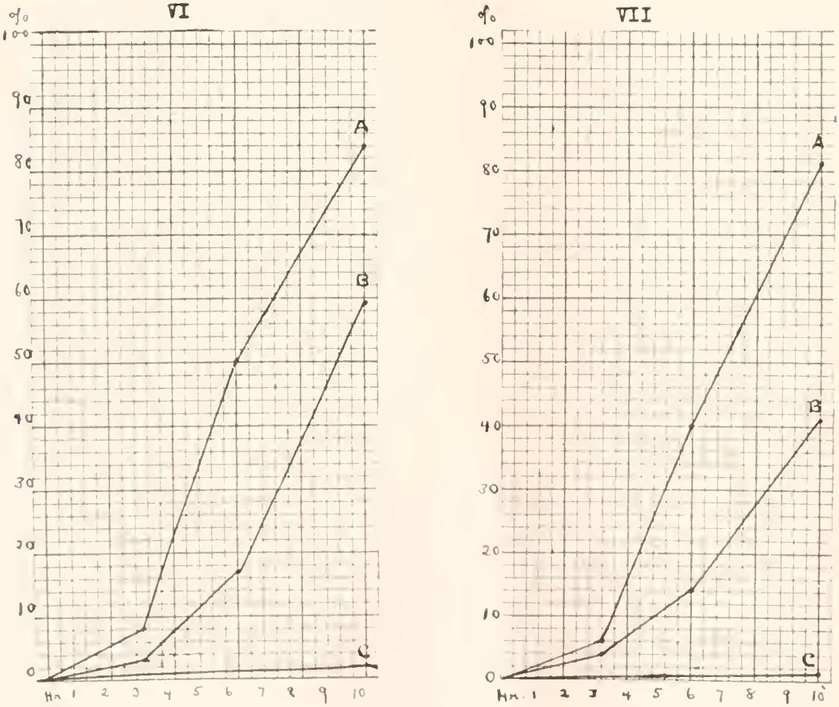
Filtrate from	Per Cent. of Ova Affected.				Per Cent. of Ova Affected.			
	4.5 Hrs.				7.5 Hrs.			
	Nor.	Abn.	Div.	Cyt.	Nor.	Abn.	Div.	Cyt.
I. Fresh Sperm	74	24.5	0.5	1	1	0	0	99
II. Heated Sperm	99	0	0	1	99	0	0	1

Nor.—normal undivided ova.

Abn.—undivided ova, abnormal in shape and in the appearance of the cytoplasm and nucleus.

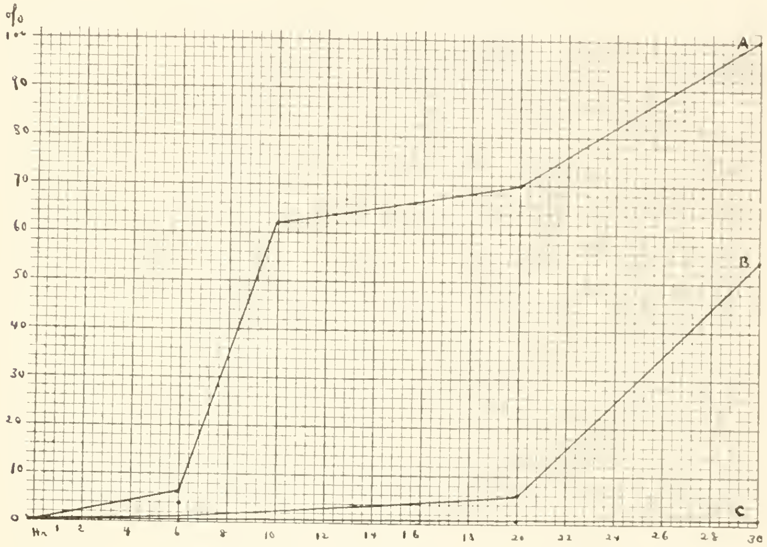
Div.—divided.

Cyt.—cytolyzed.



Graphs VI. and VII. The relation of the strength of the filtrate to the percentage of ova divided and cytolyzed—*Arbacia*. VI. Average of four experiments. VII. Average of twelve experiments. *A* = sperm filtrate from a 5 per cent. suspension. *B* = sperm filtrate from a 2 per cent. suspension. *C* = sea-water control.

Practically no heated sperm reach the surface of the filter mantle as they do not pass through the Whatman filter paper. This might appear to indicate that any active substance is adherent to the heated.coagulated sperm and not readily given off into sea-water.



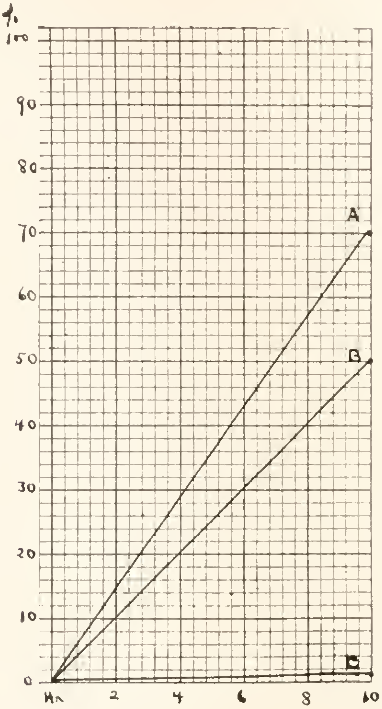
Graph VIII. Relation of the strength of the dialyzate to the percentage of ova divided and cytolyzed—*Arbacia punctulata*. A = average of three experiments with dialyzates from 50 per cent. sperm suspensions. B = average of three experiments with dialyzates from 25 per cent. sperm suspensions. C = average of six experiments with sea-water controls. Duration of dialysis = ten hours.

Another filtrate was made by filtering a heated sperm suspension directly through a Mandler filter. As a control part of the same suspension, unheated, was passed through a second filter. The effects of these two preparations are compared in Table III.

The filtrates of the heated sperm suspension have as great an effect on unfertilized ova of the same species as filtrates of fresh suspensions if the suspensions are filtered directly.

The Effect of Boiled Filtrates of Arbacia Sperm.—If the substance which so affects the eggs is an enzyme, heated filtrates may be ineffective. A filtrate prepared from a five per cent. sperm suspension was heated to boiling and then cooled rapidly.

An equal volume of sea-water was treated in the same manner and used as a control. Unheated filtrate and unheated sea-water served as additional controls. The results are given in Table IV.



Graph IX. Correlation between the duration of dialysis and the percentage of ova divided and cytolyzed—*Arbacia*. *A* = dialyzate from a 50 per cent. sperm suspension. Duration of dialysis = twenty hours. *B* = dialyzate from a 50 per cent. sperm suspension. Duration of dialysis = ten hours. *C* = sea-water dialyzed for twenty hours against sea-water.

Heating the filtrate as above indicated does not greatly affect its power to produce segmentation and cytolysis of ova of the same species. It should be emphasized that the hydrogen-ion concentration and the osmotic pressure of these solutions are like those of the sea-water controls.

It is thus evident that filtrates and dialyzates of spermatozoa of *Arbacia* contain some substance, not destroyed by heat, which produces profound changes in ova of the same species.

TABLE III.

A COMPARISON OF THE ACTION OF FILTRATES PREPARED FROM HEATED AND FROM UNHEATED SPERM SUSPENSIONS OF *Arbacia* FILTERED DIRECTLY THROUGH MANDLER FILTERS.

Exp.	Filtrate from	Per Cent. of Ova Affected.			Per Cent. of Ova Affected.		
		2 Hrs.			20 Hrs.		
		Nor.	Abn.	Cyt.	Nor.	Abn.	Cyt.
167	I. Fresh Sperm	98	0	2	0	0	100
	II. Heated Sperm	100	0	0	0	0	100
170	I. Fresh Sperm	62	0	38	0	0	100
	II. Heated Sperm	53	0	47	0	0	100

Nor.—normal undivided ova.

Abn.—undivided ova, abnormal in shape and in the appearance of cytoplasm and nucleus.

Cyt.—cytolyzed. (Partial or complete division precedes cytolysis.)

TABLE IV.

A COMPARISON OF THE ACTION OF HEATED AND UNHEATED SPERM FILTRATES ON OVA OF THE SAME SPECIES—*Arbacia punctulata.*

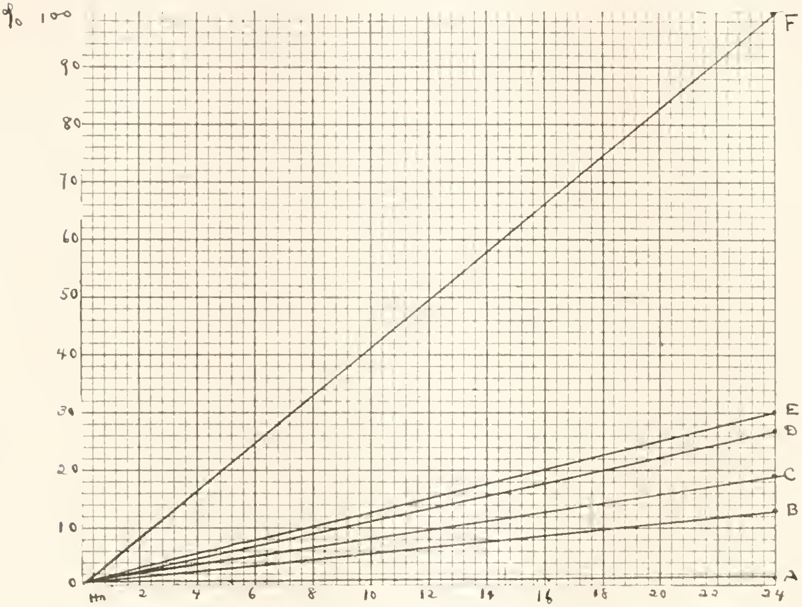
Time = 14 Hrs.	Per Cent. of Ova Affected.			Per Cent. of Ova Affected.		
	Exp. *A*—III.			Exp. *A*—IV.		
Solution.	Nor.	Div.	Cyt.	Nor.	Div.	Cyt.
Sea-water	98	0	2	99	0	1
Heated Sea-water	98	0	2	99	0	1
Sperm Filtrate	62	3	35	74	3	23
Heated Filtrate	84	0	16	81	2	17

Nor.—undivided and normal.

Div.—divided.

Cyt.—cytolyzed.

b. Strongylocentrotus purpuratus and Strongylocentrotus franciscanus.—The same methods employed with two other species of sea-urchins—*Strongylocentrotus purpuratus* and *Strongylocentrotus* *franciscanus*—at Pacific Grove, California, from January to June 1921, yielded similar results. Longer exposures were required than with ova of *Arbacia*. The ova of *S. franciscanus* were the more susceptible to the action of the sperm filtrates, but unfortunately ripe ova of this species were rare during these months of 1921 because of an extremely cold season. It is well known that the ova of *S. purpuratus* are resistant to parthenogenetic agents, Loeb (1916, pp. 99–103). The hope that the conditions of low temperature (12°–15° C.) and high hydrogen-ion concentration (p_H 7.6–7.8) prevailing at Pacific Grove might favor normal segmentation and normal development of ova treated with sperm filtrates was not realized. Yet, as will be demonstrated in later experiments, a brief exposure to such filtrates



GRAPH X. The effect of limited exposure of ova to sperm filtrates—*Arbacia*. Experiment 156—Observation at the end of twenty-four hours. The percentage of ova divided and cytolyzed is indicated. *A* = sea-water. *B* = exposure to 5 per cent.* filtrate for five minutes. *C* = exposure to 5 per cent. filtrate for ten minutes. *D* = exposure to 5 per cent. filtrate for thirty minutes. *E* = exposure to 5 per cent. filtrate for sixty minutes. *F* = exposure to 5 per cent. filtrate for twenty-four hours.

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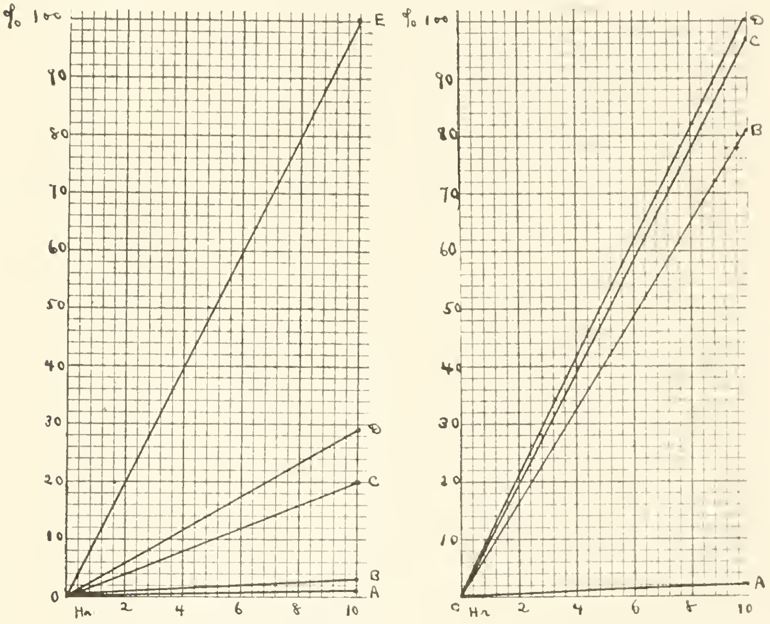
* 5 per cent. filtrate = a filtrate from a 5 per cent. suspension.

produced decided changes in ova of both species of *Strongylocentrotus*.

c. Nereis limbata.—Sperm filtrates of *Nereis limbata* produce effects on ova of this species comparable to those produced by foreign sperm filtrates. An exposure of *Nereis* eggs to filtrates made from one, two or ten per cent. *Nereis* sperm suspensions results in the formation of jelly and fertilization membranes and the complete maturation of a certain percentage of eggs. The most satisfactory results were obtained with eggs transferred directly to a two per cent. filtrate, and within two hours after



Graph XI. Graph XII.

The effect on ova of *Arbacia* of limited exposure to filtrates.

Filtrate of a 2 Per Cent. Suspension.	Filtrate of a 2 Per Cent. Suspension.
A = sea-water 10 hrs.	*A* = sea-water 10 hrs.
= filtrate 15″–45″	= filtrate 15″–45″
B = " 75″	*B* = " 210″
C = " 135″	*C* = " 4.5 hrs.
D = " 195″	*D* = " 9 hrs.
E = " 9 hrs.	

The percentages of ova divided and cytolyzed are indicated.

the *Nereis* were collected. In this experiment the majority of the eggs matured. The maturation was almost as rapid as in fertilized eggs but the subsequent segmentation and development was slow and abnormal. Segmentation into two, four, and eight cells was observed and verified for me by Dr. Alvalyn Woodward. Free swimming trochophores developed which were abnormal in shape, internal structure, and in the distribution of cilia. In no case did such changes occur in the sea-water controls. Similar results were obtained with four sperm filtrates of *Nereis limbata*.

Summary of the Effects of Sperm Filtrates and Dialyzates on Ova of the Same Species.—Sperm filtrates and dialyzates of *Arbacia punctulata*, and sperm filtrates of *Strongylocentrotus purpuratus*, of *Strongylocentrotus franciscanus*, and of *Nereis limbata* produce marked changes in ova of the same species. These are characterized by changes in form; nuclear and cell division; and, with the exception of *Nereis limbata*, by subsequent fragmentation and cytolysis. "Fragmentation" is a separation of the segments due possibly to a lack of a fertilization membrane. In *Nereis limbata* the sperm filtrates cause maturation; formation of fertilization membranes; segmentation; and, in a low percentage of cases, the development of abnormal larvæ.

V. FILTRATES AS FACTORS IN PARTHENOGENESIS.

It seemed possible in view of the results obtained by Loeb (1905) and others that the ova of sea-urchins, treated with filtrates of sperm, might develop perfectly if they were subsequently exposed for a brief period to Loeb's "hypertonic sea-water". In Loeb's experiments, exposure of unfertilized ova of sea-urchins to hypertonic sea-water, at the temperature prevailing during the breeding season, produced development in a very small percentage of the ova. Fertilization membranes did not form usually and the developing plutei did not swim at the surface of the water as did normal plutei. A brief preliminary treatment of the ova with a very dilute solution of butyric acid increased the percentage of ova which developed, and the latter were normal in macroscopic structure and in their reactions. Recently Just (1922), employing a greater concentration than that used by Loeb, has succeeded in producing healthy viable plutei of *Arbacia punctulata* by exposing ova of this species to hypertonic sea-water alone. In view of these results it may be that the butyric acid sensitizes the eggs to the action of the hypertonic sea-water. Experiments were undertaken to determine whether or not sperm filtrates may also act as sensitizers.

The procedure employed in this phase of the investigation was as follows: unfertilized eggs of *Arbacia* were exposed for periods of one minute to two hours to a sperm filtrate and subsequently for twenty to thirty-five minutes to Loeb's hypertonic sea-water. A control series consisted of ova exposed first to sea-water and then to Loeb's hypertonic sea-water. One set of unfertilized and one set of fertilized eggs served as controls for each experiment.

In the majority of experiments no development took place in the ova exposed to hypertonic sea-water only. When such an exception occurred, the percentage of ova which developed was small and the plutei did not swim at the surface of the water. In every experiment many of the ova subjected to the double treatment, *i.e.*, to the filtrate followed by the hypertonic sea-water, developed as far as the blastula and gastrula stages. A small percentage developed into perfect plutei which swam at the surface. The majority cytolyzed. Because of the high percentage which cytolyze at the time when the blastulæ and gastrulæ form, it is impossible to determine the exact percentage which develop to these stages. The sea-water containing them was centrifuged and the residue examined. An effort was made to ensure the transfer of equal quantities of eggs and of sea-water in each experiment, and equal periods of centrifuging were employed to ensure equal possibilities of precipitation of ova or of developing individuals. The results of these experiments are given in Table V.

As already indicated the sperm filtrates rarely cause membrane formation. In the few instances where these form, they are thin and but slightly elevated. In one filtrate such membranes developed on ova after an exposure to the filtrate for thirty to thirty-five minutes. Of the ova remaining in the filtrate for one to fifteen minutes and subsequently transferred to hypertonic sea-water for twenty to thirty-five minutes, a larger percentage developed than in other experiments. In this series no ova developed after exposure to hypertonic sea-water alone. In Table VI. the results of these experiments are summarized. The figures indicate approximate percentages.

TABLE V.

A COMPARISON OF THE NUMBER OF OVA OF *Arbacia* WHICH DEVELOP AFTER TREATMENT WITH FILTRATE FOLLOWED BY EXPOSURE TO HYPERTONIC SEA-WATER.

Time of observation and total count = 70 hrs.

Transfer from	Sea-water.			Hypertonic S.W. 30″			Hypertonic S.W. 35″		
	B.	G.	P.	B.	G.	P.	B.	G.	P.
Sea-water 15″	0	0	0	1	0	1	2	0	0
Filtrate									
2″	0	0	0	13	0	5	56	0	28
10″	0	0	0	0	0	2	41	0	14
15″	0	0	0	74	0	0	36	0	3

The letters B, G, and P indicate blastulæ, gastrulæ, and plutei respectively.

The figures given here indicate the number, not the percentage, of developing ova.

A comparison of the effects of the preliminary treatment with sea-water and with sperm filtrates reveals, in the latter experiments, a slight increase in the percentage of ova which develop into plutei perfect in macroscopic appearance and in reactions; and a decided increase in the percentage which cytolyze after exposure to hypertonic sea-water. The preliminary treatment with the sperm filtrate apparently sensitizes the ova to the action of the hypertonic sea-water.

Tests were also made to determine whether the sperm filtrates increase the susceptibility of the ova of *Strongylocentrotus purpuratus* to the action of hypertonic sea-water. Certain preliminary experiments were performed to familiarize the writer with the effect of hypertonic sea-water alone on the ova of this species. Ova were exposed to it for periods of thirty minutes to four hours. The effect was tested by transferring them to sea-

TABLE VI.

THE EFFECT PRODUCED ON OVA OF *Arbacia punctulata* BY SPERM FILTRATES OF THE SAME SPECIES FOLLOWED BY HYPERTONIC SEA-WATER.

Transfer from	To Hypertonic S.W. 20″.				Hypertonic S.W. 25″.				Hypertonic S.W. 30″.				Hypertonic S.W. 35″.			
	Nor.	Div.	Dev.	Cyt.	Nor.	Div.	Dev.	Cyt.	Nor.	Div.	Dev.	Cyt.	Nor.	Div.	Dev.	Cyt.
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Sea-water 15″	95.8	0	0	4.2	91	1	0	8	90	0	0	10	90	0	0.5	9.5
Filtrate 1″	75.5	0	0	24.5	53.6	0	5.7	41.7	75	0	4.5	20.5	27.5	0	1.5	71
5″	0	0	0	100	75	0	3.8	21.2	62	0	3	35	28.3	0	2	69.7
10″	66	0	3.4	30.6	8.8	0	0	91.2	21.8	0	6.1	72.1	23.6	0	0.4	76
15″	48	0	2.7	49.3	14	0	1	85.0	3	0	0	97	1	0	0	99

water and to inseminated sea-water. Ova treated for thirty minutes to two hours were uninjured. Longer exposures caused cytolysis of ova transferred to sea-water and prevented normal fertilization and development of ova subsequently inseminated in sea-water. The percentage of the ova affected bore a direct relation to the duration of the exposure to the hypertonic sea-water.

A repetition of Loeb's method of producing parthenogenesis in this species demonstrated that treatment with 1.5 per cent. butyric acid for one minute followed by hypertonic sea-water for one hour led to development of the majority of the ova. Other experiments indicated that a slight decrease in hydrogen-ion concentration (*e.g.*, p_H 8.3–8.7) facilitated the process. In place of the butyric acid ova were given a preliminary treatment with sperm filtrates in the hope of obtaining development, or of intensifying the action of the hypertonic sea-water. The results at the end of eighteen hours are indicated in Table VII.

TABLE VII.

EFFECT OF EXPOSURE TO FILTRATE FOLLOWED BY HYPERTONIC SEA-WATER ON OVA OF *Strongylocentrotus purpuratus*.

Exp.	Prep.	% Filt.	Duration of Exposure.		Effects on the Ova.			
			To		Percentage.			
83	1/26	10	Filtrate	Hyper-tonic S.W.	Nor.	Abn.	Div.	Cyt.
			0″	70″	99	0	0	1
			30″	70″	98	0	0	2
			60″	70″	98	0	0	2
			120″	70″	66	0	1	33
			30″	120″	99	0	0	1
			60″	120″	0	97.5	0	2.5
			120″	120″	0	0	0	100

It has already been mentioned that ova of *Strongylocentrotus purpuratus* are more resistant to parthenogenetic agents than are those of *Arbacia punctulata*. In these experiments ova of the former species, exposed either to hypertonic sea-water alone or to filtrate and hypertonic sea-water, failed to develop. Occasionally atypical membranes formed after the double treatment and irregular division ensued. It is of interest that ova which had been exposed to the filtrate for two hours did not shrink when transferred to hypertonic sea-water, as did those transferred directly from sea-water to the hypertonic sea-water. This would seem to indicate that the permeability of the ova exposed to the filtrate had increased. Another indication of the effect of the filtrate in these experiments follows: Ova given the double treatment of filtrate followed by hypertonic sea-water were less capable of normal fertilization and development than those exposed to hypertonic sea-water alone.

Summary of the Function of Filtrates as Factors in Parthenogenesis.—Exposure of ova of *Arbacia punctulata* or of *Strongylocentrotus purpuratus* to sperm filtrates of the same species produce changes which render them more susceptible to the action of hypertonic sea-water. This is indicated in *Arbacia* by a larger percentage of development and of cytolysis; and in *S. purpuratus* by a greater tendency of ova to cytolyze or to lose their power of normal fertilization and development if exposed to both filtrate and hypertonic sea-water.

VI. Filtrates as Factors in Fertilization.

The increase produced by sperm filtrates in the susceptibility of *Arbacia* ova to parthenogenetic agents, and the apparent increase in permeability of ova of *S. purpuratus* suggest that such preparations may also facilitate fertilization.

An attempt was made to obtain "resistant" eggs early in the breeding season in order to try the effect of fertilizing such ova in sperm filtrates. During two seasons such "resistant" eggs were not found. The ova obtained were either immature, as indicated by their failure to develop if fertilized with a small or large amount of sperm suspension, or else ripe. In the latter case no increase over the usual percentage of development could be expected.

Conditions leading to increase in permeability of mature ova tend to allow the entrance of more than a single spermatozoön when such eggs are inseminated. This polyspermy usually results in abnormal development. It may occur if mature ova are inseminated in sperm filtrates. To determine this, two milliliters of washed *Arbacia* eggs were placed in twenty-five milliliters of sperm filtrate and, as a control, a similar quantity in twenty-five milliliters of sea-water. These were inseminated immediately, or after exposure to the filtrate or sea-water for from one minute to four hours. Frequent observations were made to determine whether the filtrates affect either fertilization or development. The effects observed are as follows in the ova fertilized in the filtrate and allowed to develop in it: lack of a normal fertilization membrane; abnormal changes in shape; a low percentage of dividing ova; abnormal division; delayed and abnormal development; and cytolysis. In the controls normal fertilization and development occurred. Similar results were obtained in thirteen series of experiments in which several filtrates were used. Gemmil (1900) describes similar conditions resulting from heavy insemination and finds them accompanying polyspermy as demonstrated by histological examination.

The abnormal development which occurs when ova are inseminated in the sperm filtrates may be a result of an abnormal fertilization reaction. If this is due to changes in the ova in the filtrate, it may be that such changes are reversible if the exposure to the filtrate is of short duration. Ova of *Arbacia punctulata* were exposed to filtrates made from two per cent. suspensions for from one minute to two and a half hours, and to filtrates from five per cent. suspensions for one to thirty minutes. Some were inseminated in the filtrates and others after transfer to sea-water. Few of those inseminated in the filtrate developed normally. The majority of those inseminated after transfer to sea-water formed fertilization membranes and developed normally. The changes produced in the ova by the filtrates, which tend to prevent normal reactions between ova and sperm, are reversible if the period of exposure is brief. Prolonged exposure to filtrates, however, wrought such changes in the majority of ova that they lost their capacity for fertilization and development completely.

Ova of *Strongylocentrotus purpuratus* exposed for short periods to sperm filtrates may not exhibit any change in optical appearance or in shape yet may be so influenced that after transfer to sea-water they fail to develop normally if inseminated with fresh sperm. Such was found to be the case in eighteen experiments in which ova were exposed to the action of a number of filtrates and subsequently inseminated in sea-water. Although the sperm were active in the filtrates, few membranes formed and these were abnormal in that they were irregular and but slightly raised from the surface of the egg. Subsequent divisions were irregular; development was slow and abnormal; and the majority of ova cytolyzed later. Gastrulæ formed in a few, but these lacked an enteron, and plutei were irregular in shape with thickened areas not normally present. These resembled plutei obtained when ova are inseminated with a large excess of sperm when polyspermy is known to occur.

A correlation between the reversibility of changes in ova produced by abnormal constituents in sea-water and the duration of their exposure to these substances has been recorded by Loeb (1915) for butyric acid and hypertonic sea-water; by Lillie (1921) for copper; and by Clowes and Smith (1923 and 1924) for hydrogen ions. In some instances the change is of such a nature that it acts as a block to the entrance of sperm; in others it permits polyspermy. Just (1923) found that eggs of *Echinarachnius* fertilized in blood, though they fail to develop, nevertheless take in sperm.

As stated by Clowes (1924) "it is difficult to distinguish polyspermic from abnormally dividing eggs without cytological examination." Such a study of ova of *Arbacia* inseminated in the sperm filtrates reveals that polyspermy occurred in many of the ova.

The interference with the fertilization process may be due in part to injury to the sperm caused by the sperm filtrate. However, examinations of suspensions of sperm in filtrates reveal that the sperm remain active in such suspensions for hours. Furthermore if such sperm are used for insemination of ova in sea-water, they will effect normal fertilization and development. The results tabulated below indicate that the sperm are not injured by an exposure of one hour either to sea-water or to a sperm filtrate in the concentrations employed; but that ova were affected by a similar duration of exposure to some of the same filtrate.

TABLE VIII.

A COMPARISON OF THE ACTION OF FILTRATES ON THE FERTILIZING CAPACITY OF SPERM AND OF OVA OF *Strongylocentrotus purpuratus*.

Exp.	Prep.	Filt.	Expos.	Ova from Sea-water.				Ova from Sperm Filtrate.			
73	12/28/I	10%		% Nor.	% Div.	% Abn. Div.	% Cyt.	% Nor.	% Div.	% Abn. Div.	% Cyt.
Sperm exposed to sperm filtrate. .			0″	0	90	10	0	0	90	10	0
			1″	0	94	3	3	0	59	35	6
			5″	0	95	5	0	0	85	10	5
			10″	0	91	5	4	0	68	29	3
			20″	0	95	3	2	0	56	43	1
			30″	0	96	3	1	3	23	18	56
			60″	0	98	2	0	0	0	30	70
Sperm suspension in sea-water. . . .			30″	0	98	1	1	0	0	33.4	66.6
			60″	0	96	0	4	0	0	12	88

It is also possible that filtrates may interfere with the development of fertilized ova. This proved to be true. The ova of *Arbacia punctulata* and of *Strongylocentrotus purpuratus* transferred ten minutes after insemination in sea-water to sperm filtrates failed to develop normally, and within eighteen hours the majority had cytolyzed.

Summary of the Action of Filtrates as Factors in Fertilization.—Sperm filtrates produce changes in the eggs of the same species which interfere with a normal fertilization reaction if the eggs are inseminated in the filtrate. The changes produced by a brief exposure are reversible; by a longer exposure irreversible. Such filtrates prevent normal development of eggs previously inseminated in sea-water. The changes are of such a nature that the entrance of sperm is facilitated and polyspermy results.

VII. TEST OF FILTRATES FOR A "SPERM-FERTILIZING AND AGGLUTINABLE SUBSTANCE."

Lillie (1919) has suggested that the spermatozoa bear a fertilizing substance identical with the agglutinable substance which is apparently lost by spermatozoa in staling in sea-water. If this is present in sea-water filtrates and dialyzates, it should combine with the "agglutinin" of "egg-water." This was tested as follows. The agglutinating unit strength of *Arbacia* egg-water was first determined, Lillie (1914). Dilutions of such egg-water with sperm filtrates and with sea-water were compared as to their power to agglutinate fresh sperm suspensions and no difference could be detected in the capacity of the two sets of dilutions to agglutinate sperm. Either an insufficient amount of the combining substance is present in the sperm filtrates or the substance or substances present do not have the power to combine with the agglutinin of the egg-water.[4]

The agglutinin does not pass through collodion sacs. If the substance in sperm dialyzates is the substance postulated by Lillie, egg-water dialyzed against a sperm suspension should lose its agglutinating power more rapidly than a similar egg-water dialyzed against sea-water. In experiments devised to test this theory no difference in the rate of loss of agglutinin could be detected.

The "fertilizin" of Lillie in *Arbacia* egg-water may, according to Woodward (1918), consist of two parts: an agglutinating and an activating substance. The latter only passes through a Mandler filter. This activates sperm of the same species, and has the power of causing parthenogenetic development of ova of *Nereis limbata*, Sampson (unpublished, quoted by Woodward) and Woodward (1921). If its action is intensified by a fertilizing substance given off by sperm, a combination of filtrates of sperm and of egg-water should be more effective than either alone. No such intensifying effect could be demonstrated in any of the experiments devised to test this possibility.

VIII. Discussion.

In *Nereis limbata* and in sea-urchins the fertilizable period of the gametes is short. Causes for the brevity of this period have been discovered for ova but not for spermatozoa, as indicated by Lillie and Just (1924) in their recent survey of the subject.

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[4] It should be recalled that some of the sperm remaining on the surface of the filter mantles and in the dialyzing sacs are agglutinable and retain their fertilizing power at the end of the periods of filtration and of dialysis.

The fact that loss of fertilizing power of the sperm occurs rapidly and precedes loss of motility has suggested the idea that the spermatozoön carries a fertilizing substance which may be lost in sea-water; and this has led to various attempts, of which this is the most recent, to isolate such a substance and to produce development of ova of the same species with it. In this investigation filtrates and dialyzates of sperm suspensions in sea-water have been obtained which initiate development of specific ova, although the development is incomplete.

It is not surprising to find that concentrated sperm suspensions exposed to egg-secretions of a foreign species, that extracts of cells, egg-secretions, and blood cause cytolysis of alien ova, "since it is recognized that something present in mammalian blood serum cytolyzes cells of unrelated animals," Loeb (1913). Sperm killed by heat, extracts of cells, and blood serum have no activating effect on ova of the same species. Specific egg-secretion ("egg-water") has no effect on ova of *Nereis*, but according to Glaser (1915) and Woodward (1918) does produce incomplete activation of ova of *Asterias* and *Arbacia*. Prolonged exposure of ova is necessary, and Lillie and Just (1924) have suggested that there are extraneous parthenogenetic factors present in the egg-water employed.

Careful tests of all the sperm filtrates and dialyzates used in this investigation indicate that the preparations do not contain living sperm or fragments of them. The ova employed are not normally parthenogenetic. Controls give evidence that no substance derived from filters or dialyzers is accountable for the results obtained; and factors which might produce parthenogenesis: abnormal specific gravity, abnormal hydrogen ion concentration, and excess of carbon dioxide, are lacking. The preparations contain carbon but insufficient nitrogen to be detected even by microchemical methods. No lipolytic enzyme could be detected. The preparations are not colloidal; and all attempts to obtain precipitates from them by means of alcohol or the reagents used by Robertson (*loc. cit.*) and Woodward (*loc. cit.*) failed. Sperm filtrates and dialyzates activate ova rapidly and this property is not destroyed by boiling. The effect produced is evidently due to a special physiological activator derived from sperm of the same species.

Experiments to determine whether the substance or substances present in filtrates and dialyzates are "tissue specific," acting only on ova, have not been undertaken. It is true that they do not cytolyze species sperm, as the latter retain their fertilizing power after prolonged exposure to such preparations. The latter are not "species specific" since they readily activate ova of unrelated species. Tests have not been performed to determine whether the effect on species and on foreign ova is due to the same constituent of the preparations.

If the substance acts as a superficial cytolytic agent as suggested by Loeb (1916), it is to be expected that membrane elevation or swelling will occur in ova of the same species exposed to the sperm filtrates and dialyzates. Such occurred in *Nereis* only. However, such preparations cause partial activation and changes in the protoplasm of sea-urchin ova; and there is evidence that the properties of the egg surface are affected. Thus, after exposure to sperm filtrates, unfertilized ova of sea-urchins are more susceptible to the action of hypertonic sea-water; are in a condition which facilitates polyspermy; and their permeability is increased. Fertilized eggs, transferred to such preparations within ten minutes after insemination in sea-water, fail to develop normally. This may be due in part at least to an increase in the permeability of the egg surface. The sperm are uninjured by long exposure and the changes in the ova are reversible if the duration of exposure to such preparations is brief.

According to the "fertilizin" theory of Lillie (1914), substances ("receptors") given off by sperm activate "fertilizin" an essential constituent of the cortex of mature eggs. This in turn initiates the development of the egg. If "receptors" exist in active form and in sufficient quantity in these sperm filtrates and dialyzates, the latter should produce the following effects: initiation of development of mature ova of the same species; such activation of "fertilizin" in egg-water as to make the latter an efficient parthenogenetic agent; such combination with an agglutinating substance in egg-water as to destroy the power of the latter to agglutinate fresh sperm suspensions. The first of these results only has been obtained in this investigation. If sperm receptors are released more readily in specific egg-water, filtrates of sperm suspensions in egg-water may evince greater activating power than a combination consisting of a sperm filtrate and filtered egg-water. Tests gave no indication of any difference between them. It is also possible, as suggested by Lillie (1915), that other substances are extracted from sperm which may tend to neutralize the activating substances released by them.

There is abundant evidence that the sperm in contact with mature ova, or secretions of ova, of the same species undergo changes which are essential for fertilization. Their metabolism is increased as indicated by their increased motility; their chemical composition is changed as indicated by decrease in refringibility, by swelling of the sperm head, by changes in viscosity, and by surface changes which permit agglutination to occur. Spermatozoa may enter unripe eggs which lack "fertilizin" or mature eggs from which it has been removed experimentally but the sperm are not changed and they do not activate the eggs.

It is significant that in order for fertilization to occur *these essential changes in the spermatozoön must be produced when the latter is in close proximity to an ovum.* Sperm which have received a long exposure to sea-water or a brief exposure to egg-water are active and may surround or even enter ova of the same species, yet fail to fertilize them. This loss of fertilizing capacity, the transitory nature of agglutination, and the inability to obtain a second agglutination reaction with sperm are indications of a loss of substances essential in fertilization. To effect perfect development *such substances must be concentrated at the surface of the sperm head at the instant when the latter comes into contact with an ovum.* Under such conditions they may initiate a chain of chemical reactions, starting in the cortex of the ovum and eventually involving all parts of the protoplasm of the egg, *i.e.*, they may activate "fertilizin." If released into sea-water or egg-water normally, or under the experimental conditions of filtration and dialysis, these substances may be unable to produce complete activation of ova of the same species, because of dilution, instability, or neutralization by other substances eliminated or extracted from sperm. They may, however, produce changes in the surface of the ovum which facilitate its reaction with a spermatozoön. These changes may account, in part, for polyspermy when ova are inseminated with an excess of fresh sperm and for the effectiveness of stale sperm if concentrated suspensions are used. Such substances may account for the antagonistic effect produced by sperm of one species on those of another; and for the neutralizing effect of concentrated sperm suspensions on the action of blood serum.

IX. Summary.

1. Solutions obtained by filtration and dialysis of suspensions of living sperm in sea-water activate ova of the same species.

2. Tests indicate that the effect is produced by some substance derived from the sperm, and not by some extraneous parthenogenetic factor.

3. Ova of *Nereis* exposed to specific sperm filtrates form fertilization membranes, complete their maturation and some develop into abnormal trochophores. Ova of sea-urchins fail to form membranes but do undergo nuclear and cell division.

4. Ova exposed to filtrates and dialyzates are rendered more susceptible to the action of hypertonic sea-water, and to the entrance of sperm.

5. In normal fertilization sperm exposed to "fertilizin" undergo profound modification in chemical structure and organization; and unless such modification occurs the sperm fail to fertilize the egg, even though they may enter it. It is possible that substances localized in the surface of the sperm head activate the ovum. Such localization is transitory.

6. Such substances, when given off by sperm into sea-water or egg-water, either because of dilution, decomposition, or admixture with waste products given off by sperm, produce definite changes in such ova, but are unable to effect complete activation of ova of the same species.

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IS THE INSECT METAMORPHOSIS INFLUENCED BY THYROID FEEDING?

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(With 5 Tables.)

The investigations on the influence of the thyroid gland on the rate of metamorphosis in insects have not as yet yielded any decisive results. Northrop ('17) fed larvæ of *Drosophila* on thyroid and ascertained that this gland is not adequate as food for the examined animals. Kunkel ('18) who studied the influence of the thyroid on the development of the fly *Lucilia*, noticed an abbreviation of the pupal stage and a retardation of growth. Abderhalden ('19) irrigated *Euphorbia* plants with 1 per cent. thyroid extract and observed that the caterpillars of *Deilephila euphorbiæ* fed on these plants were "zum Teil auffallend klein." In the researches of Romeis and v. Dobkiewicz ('20) the larvæ of the flies *Calliphora vomitoria* fed on thyroid gland underwent pupation later than the control specimens, their weight, however, being not smaller. From his experiments Kahn ('21) draws the conclusion that in *Corethra plumicornis*, *Ecdyurus forcipula* and *Tenebrio molitor* neither the human thyroid nor the preparation "Jodalbacid" have any influence on the metamorphosis of their larvæ.

The discrepancy of the above results may be explained not only by the heterogeneity of the material of animals and of the substances used, but, at least to the same degree, by the probably too scanty material in separate experiments as well as by the insufficient method of the elaboration of the data recorded. *E.g.*, Abderhalden's opinion mentioned above is not supported by any numerical data and can consequently not be considered as decisive from the biometrical standpoint, all the more as we do not know the pedigrees of the animals under examination.

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[1] Paper from the Laboratory of Experimental Morphology., cf. *Mém. de l'Institut National Polonais d'Économie Rurale à Pulawy*, Vol. 5, 1924, presented at print September 22, 1924.

Similarly, we cannot, according to my view, consider as decisive the results obtained by Kahn who relies on 16 *Tenebrio* larvæ of which 6 were kept without any food, and every two specimens from the remaining 10 were fed differently (fresh meat, fresh thyroid, dried thyroid, meal and "Jodalbacid"). The experiments of the same author on *Corethra* and *Ecdyurus* were made on specimens collected in natural waters and therefore on genetically totally unknown materials. Similar relations are often met with also in other problems referring to the glands of internal secretion. In these cases genetically homogeneous animals used for the experiments and statistical elaboration of adequate data, would, according to my opinion, contribute to set aside numerous discrepancies.

My experiments have been made on caterpillars from 4 lots of eggs of *Lymantria dispar* L. deriving from 4 brother-sister matings of moths belonging to the same pedigree and reared by the author for several years. In order to deal with materials homogeneous also in respect to age, I selected exclusively caterpillars which hatched during the night between April 27th and 28th. On June 5th the thoroughly mixed caterpillars of each lot were divided at random in two parts, one of which served as control, the other being fed on leaves with thyroid. Fresh willow twigs were accurately sprinkled with water solution of the "Tablettæ thyreoideæ" from the factory of "Gedeon Richter" in Budapest. Ca. 450 experimental caterpillars received daily: in the beginning 2, from June 12th 3, from June 18th 4 tabloids, equal to 1.0, 1.5 and 2.0 grams respectively of fresh gland. (The efficacy of the preparation had been previously tested on tadpoles of *Rana temporaria*.) The control animals were fed on willow twigs of the same species, sprinkled with pure water. In both cases the leaves were administered daily (after the superfluous water had been dried) in slight excess of requirements, so that the whole food was almost totally consumed by the next day. The thyroid-fed caterpillars took food as readily as those of the control.

All animals were bred in pasteboard boxes of identical size at a temperature of from 14° to 19° C. Each chrysalid being kept separately, the duration of the pupal period could be established for each specimen. Pupation as well as emergence of moths was checked every day between 6 and 6:15 o'clock in the evening. The chrysalids were weighed immediately afterwards, so that the age of the weighed pupæ ranged from 0 to 24 hours. The data obtained were biometrically studied according to the commonly used formulæ. On account of the remarkable sexual dimorphism the data were collected for either sex separately. The total number of pupæ was 818, of which 234 male and 188 female were thyroid fed specimens and 171 male and 225 female control animals. Emergence occurred from 782 chrysalids, viz. 218, 182, 161 and 221 specimens respectively (cf. the number of animals in separate experiments in Tables I. and II).

The first chrysalis of the whole material appeared on June 24th. The average terms of pupation of the thyroid-fed and of the control caterpillars calculated in relation to the above term of the "first chrysalis" are recorded in Table I. We notice that the observable differences of the terms of control and experimental material were positive only in 3 cases, in the remaining 5 being on the contrary negative. The difference was here biometrically significant exclusively in the males of the second experiment, the ratio of the difference to its probable error in this case only approximating the required number 4. Moreover, on surveying the data referring to the duration of the pupal period (Table II.) we observe that here too the difference between the control and the thyroid-fed animals is not always positive, the ratio of the difference to their probable errors oscillating only between 0.05 and 2.5 and therefore being in no case significant. This indicates that neither the rate of the hystolytical processes in the caterpillars nor the rate of development in the chrysalids were in our experiments influenced by the thyroid added to the food. In other words, this substance did not elicit any changes in the course of metamorphosis of the examined insects.

From the theoretical point of view this result requires however the following remarks. It has often been emphasized that the influence of glands of internal secretion may be not only of various intensity, but even essentially different in relation to the developmental or growth stage of the animals experimented

TABLE I.

Data Concerning the Term of Pupation of the Caterpillars of *Lymantria dispar* L., in Days.

The term of pupation calculated from the day of the appearance of the first pupa in the whole material, *i.e.* from June 24.

n, number of specimens; σ, standard deviation; $A \pm E_A$, average term of pupation with its probable error; $v \pm E_v$, coëfficient of variability of this term together with its probable error; Diff. $\pm E_{\text{Diff.}}$, difference together with its probable error; Diff./$E_{\text{Diff.}}$, ratio of the difference to its probable error.

Sex.	Number Corresponding to Each Experiment.	Material of Caterpillars.	n.	$\pm\sigma$.	$A \pm E_A$.	Difference between b and a in Averages.		$v \pm E_v$.	Difference between b and a in Coëfficients of Variability.	
						Diff. $\pm E_{\text{Diff.}}$	Diff./$E_{\text{Diff.}}$		Diff. $\pm E_{\text{Diff.}}$	Diff./$E_{\text{Diff.}}$
Males	1	(*a*) "Thyroid" .... (*b*) Control.......	59 45	3.17 2.19	8.98 ± 0.28 8.62 ± 0.22	−0.36 ± 0.36	1.0	35.3 ± 2.45 25.4 ± 1.92	−9.9 ± 3.11	3.2
	2	(*a*) "Thyroid" .... (*b*) Control.......	68 58	2.13 1.77	6.21 ± 0.17 7.07 ± 0.16	+0.86 ± 0.23	3.7	34.3 ± 2.21 25.0 ± 1.66	−9.3 ± 2.76	3.4
	3	(*a*) "Thyroid" .... (*b*) Control.......	50 39	2.57 2.53	10.32 ± 0.25 10.56 ± 0.27	+0.24 ± 0.37	0.6	24.9 ± 1.78 24.0 ± 1.94	−0.9 ± 2.63	0.3
	4	(*a*) "Thyroid" .... (*b*) Control.......	57 29	2.61 2.32	6.81 ± 0.23 5.72 ± 0.29	−1.09 ± 0.37	2.9	38.3 ± 2.75 40.6 ± 4.15	+2.3 ± 4.98	0.5
Females	1	(*a*) "Thyroid" .... (*b*) Control.......	55 64	3.58 3.95	13.20 ± 0.33 13.57 ± 0.33	+0.37 ± 0.47	0.8	27.1 ± 1.87 29.1 ± 1.88	+2.0 ± 2.65	0.8
	2	(*a*) "Thyroid" .... (*b*) Control.......	48 59	2.29 2.65	11.96 ± 0.22 11.15 ± 0.23	−0.81 ± 0.32	2.5	19.1 ± 1.36 23.8 ± 1.56	+4.7 ± 2.07	2.3
	3	(*a*) "Thyroid" .... (*b*) Control.......	37 52	3.46 3.35	14.92 ± 0.38 14.19 ± 0.31	−0.73 ± 0.49	1.5	23.2 ± 1.91 23.6 ± 1.65	+0.4 ± 2.52	0.2
	4	(*a*) "Thyroid" .... (*b*) Control.......	48 50	4.41 3.99	11.96 ± 6.43 10.24 ± 0.38	−1.72 ± 0.57	3.0	36.9 ± 2.87 39.0 ± 3.00	+2.1 ± 4.15	0.5

TABLE II.

Data Concerning the Duration of Pupal Period of *Lymantria dispar* L., in Days.

n, number of specimens; σ, standard deviation; $A \pm E_A$, average duration together with its probable error; $v \pm E_v$, coëfficient of variability of the duration together with its probable error; Diff. $\pm E_{\text{Diff.}}$, difference together with its probable error; Diff./$E_{\text{Diff.}}$, ratio of the difference to its probable error.

Sex.	Number Corresponding to Each Experiment.	Material of Chrysalids.	n.	$\pm\sigma$.	$A \pm E_A$.	Difference between b and a in Averages.		$v \pm E_v$.	Difference between b and a in Coëfficients of Variability.	
						Diff. $\pm E_{\text{Diff.}}$	Diff./$E_{\text{Diff.}}$		Diff. $\pm E_{\text{Diff.}}$	Diff./$E_{\text{Diff.}}$
Males	1	(*a*) "Thyroid" .... (*b*) Control.......	57 42	1.37 1.19	17.18 ± 0.12 17.33 ± 0.12	+0.15 ± 0.17	0.9	7.97 ± 0.50 6.87 ± 0.51	−1.10 ± 0.71	1.5
	2	(*a*) "Thyroid" .... (*b*) Control.......	65 57	1.21 0.88	17.15 ± 0.08 17.25 ± 0.08	+0.10 ± 0.11	0.9	7.06 ± 0.42 5.10 ± 0.32	−1.96 ± 0.53	3.7
	3	(*a*) "Thyroid" .... (*b*) Control.......	46 35	1.12 1.43	18.72 ± 0.11 19.00 ± 0.16	+0.28 ± 0.19	1.5	5.98 ± 0.42 7.53 ± 0.61	+1.55 ± 0.74	2.1
	4	(*a*) "Thyroid" .... (*b*) Control.......	50 27	1.18 1.13	16.58 ± 0.11 16.59 ± 0.15	+0.01 ± 0.19	0.05	7.12 ± 0.48 6.81 ± 0.62	−0.31 ± 0.78	0.4
Females	1	(*a*) "Thyroid" .... (*b*) Control.......	53 63	1.54 1.45	15.87 ± 0.14 16.03 ± 0.12	+0.16 ± 0.18	0.9	9.70 ± 0.64 9.05 ± 0.54	−0.65 ± 0.84	0.8
	2	(2) "Thyroid" .... (*b*) Control........	47 59	1.17 1.42	15.89 ± 0.12 15.80 ± 0.12	−0.09 ± 0.17	0.5	7.36 ± 0.51 8.99 ± 0.56	+1.63 ± 0.76	2.1
	3	(*a*) "Thyroid" .... (*b*) Control.......	34 50	1.31 1.08	16.65 ± 0.15 17.10 ± 0.10	+0.45 ± 0.18	2.5	7.87 ± 0.64 6.32 ± 0.43	−1.55 ± 0.77	2.0
	4	(*a*) "Thyroid . ." .. (*b*) Control.......	48 49	1.71 1.55	15.58 ± 0.17 15.39 ± 0.15	−0.19 ± 0.23	0.8	10.98 ± 0.76 10.07 ± 0.69	−0.91 ± 1.03	0.9

upon (cf., *e.g.*, the manual of Biedl, '22, as well as the papers of Romeis, '14–'15, '16 and '18, Gedroyć, '23, Deutsch, '23, and others). Consequently the absence of any considerable and constant differences in the processes of metamorphosis between my control animals and those fed on thyroid, does not solve our problem. We ought to take into consideration, that owing to individual fluctuations on the starting day of the thyroid feeding, *i.e.* on June 5th, the larvæ experimented upon without any doubt differed from one another considerably both in regard to weight and most probably also to stage of development, although having hatched during one and the same night. It therefore was a priori not known whether the average terms of metamorphosis remained unchanged in spite of the different influence exerted by thyroid on separate specimens. For instance, if the positive and the negative influence exerted by thyroid feeding on separate caterpillars were of identical intensity, the average duration of the larval as well as of the pupal stage would remain unchanged. The so to speak latent influence of thyroid feeding ought to be noticeable here only by the variation of the terms of metamorphosis, as the range of individual fluctuations of the terms of pupation and emergence of adult moths would in such case undergo in the experimental materials a distinct widening. As a matter of fact in my experiments no remarkable changes in the coefficients of variability of these terms of the mentioned variation may be seen (cf. Tables I. and II.). The differences of the appertaining coefficients between control and thyroid materials have opposite signs, in one case being positive, in the remaining negative. The ratio of the difference between the coefficients to its probable error was only in few cases larger than 3, as a rule attaining from 0.2 to 2.3 only. It follows therefrom that these biometrical tests show no distinct influence of thyroid feeding on the rate of metamorphosis of the insects experimented.

The above results require a certain supplement in still another direction. Viz: the question arises whether in these experiments the lack of influence of thyroid on metamorphosis is caused by too small quantities of the substance added, or unsuitable age of the animals at the beginning of the experiments. Decisive answers to this question have been attained, according to my view, by weighing fresh pupæ. It appeared that the growth of the caterpillars (the term of which was determined by the weight of fresh chrysalids) underwent in all cases, without any exception, a considerable retardation under the influence of thyroid feeding (cf. Table III.). The differences between the control and the experimental material are here always positive, in 3 cases being biometrically very significant. The thyroid had therefore in this respect an undoubtedly negative influence on the processes of larval growth, *i.e.*, the dosing of thyroid as well as the age of the animals have been well chosen to demonstrate the supposed influence of this substance. It is very characteristic that the variability of weight of the "thyroid" caterpillars does not undergo any essential and corresponding changes (cf. Table III.). This points to the fact that the negative influence exerted here by thyroid feeding on growth of caterpillars had the same qualitative and quantitative effect for all animals.

The influence of organic or even inorganic compounds of iodine on amphibian metamorphosis is much discussed in recent years and the problem contains certain discrepancies (cf., *e.g.*, the papers of Romeis, '18, Allen, '19, Hirschler, '18–'19 and '22, Huxley, '22, Huxley and Hogben, '22, Uhlenhuth, '22, and others). Gedroyć ('23) studied the problem in respect to insects, viz. on *Deilephila euphorbiæ* L. In two experiments each containing 5 "iodine" caterpillars, the author observed an acceleration of the pupation of the experimental specimens relatively to the 15 control caterpillars. On the contrary, in the third analogous experiment in which also 5 animals, but 2 or 4 days younger than the larvæ of the preceding series, were used, the larval period underwent in the "iodine" material even a certain prolongation. Hence the inference of Gedroyć as to the decisive influence of age and "state of growth" of the animals exerted on the direction of the action of iodine.

The material of Gedroyć being scanty I have studied anew the problem of the influence of iodine on insect metamorphosis. My experiments were performed on caterpillars of *Pieris brassicæ* L. Larvæ hatched between August 2d and 3d were used in all 4 experiments. Cabbage leaves abundantly sprayed with Lugol's

TABLE III.

Data Concerning the Weight of Chrysalids of *Lymantria dispar* L., in Mg.

n, number of specimens; σ, standard deviation; $A \pm E_A$, average weight together with its probable error; $v \pm E_v$, coëfficient of variability of the weight together with its probable error; Diff. $\pm E_{\text{Diff.}}$, difference together with its probable error; Diff./$E_{\text{Diff.}}$, ratio of the difference to its probable error.

Sex.	Number Corresponding to Each Experiment.	Material of Chrysalids.	n.	$\pm\sigma$.	$A \pm E_A$.	Difference between b and a in Averages.		$v \pm E_v$.	Difference between b and a in Coëfficients of Variability.	
						Diff. $\pm E_{\text{Diff.}}$	Diff./$E_{\text{Diff.}}$		Diff. $\pm E_{\text{Diff.}}$	Diff./$E_{\text{Diff.}}$
Males	1	(*a*) "Thyroid"	59	70.6	367.0 ± 6.2	+14.1 ± 10.7	1.3	19.2 ± 1.24	+3.6 ± 2.10	1.7
		(*b*) Control.......	45	86.8	381.1 ± 8.7			22.8 ± 1.70		
	2	(*a*) "Thyroid"	68	69.5	408.8 ± 5.7	+18.8 ± 9.4	2.0	17.0 ± 1.01	+2.7 ± 1.63	1.7
		(*b*) Control.......	58	84.3	427.6 ± 7.5			19.7 ± 1.28		
	3	(*a*) "Thyroid"	50	89.9	428.0 ± 8.6	+11.7 ± 12.4	0.9	21.0 ± 1.48	−2.3 ± 2.09	1.1
		(*b*) Control.......	39	82.2	439.7 ± 8.9			18.7 ± 1.48		
	4	(*a*) "Thyroid"	57	79.6	430.7 ± 7.1	+60.7 ± 13.5	4.5	18.5 ± 1.21	+0.2 ± 2.09	0.1
		(*b*) Control.......	29	92.1	491.4 ± 11.5			18.7 ± 1.71		
Females	1	(*a*) "Thyroid"	55	248.4	1078.2 ± 22.6	+90.6 ± 32.5	2.8	23.0 ± 1.56	+0.7 ± 2.16	0.3
		(*b*) Control.......	64	277.4	1168.8 ± 23.4			23.7 ± 1.49		
	2	(*a*) "Thyroid"	48	299.6	1383.4 ± 29.2	+92.8 ± 42.4	2.2	21.7 ± 1.56	+2.0 ± 2.20	0.9
		(*b*) Control.......	59	350.5	1476.2 ± 30.8			23.7 ± 1.55		
	3	(*a*) "Thyroid"	37	307.8	1175.6 ± 34.1	+216.6 ± 46.6	4.6	26.8 ± 2.25	−2.4 ± 2.83	0.8
		(*b*) Control.......	52	340.3	1392.2 ± 31.8			24.4 ± 1.71		
	4	(*a*) "Thyroid"	48	237.7	1187.6 ± 23.1	+440.4 ± 41.4	10.6	20.0 ± 1.43	+2.1 ± 2.12	1.0
		(*b*) Control.......	50	359.3	1628.0 ± 34.3			22.1 ± 1.56		

solution (6 g. IK, 4 g. I in 100 g. H_2O) were daily administered to the caterpillars of experiments 1 and 2 from August 16th, and in those of experiments 3 and 4 from August 22d. Leaves designed for control animals were similarly changed daily and sprayed with water. All animals were kept under glass vessels. The administered leaves were taken by the "iodine" caterpillars as well as by those of control materials. Data referring to the pupation *term* of these caterpillars as well as to the average *weight* of the resulting chrysalids are recorded in Table IV.

TABLE IV.

Data Concerning the Term of Pupation and the Weight of Pupæ from "Iodine" Caterpillars of *Pieris brassicæ* L.

The terms of pupation calculated from the beginning of iodine feeding.

Number Corresponding to Each Experiment.	Material of Specimens.	Number of Specimens.	The Average Term of Pupation, in Days.	Difference between *b* and *a*.	The Average Weight of Chrysalids, in mg.	Difference between *b* and *a*.
1	(*a*) "Iodine" . .	12	12.2	− 2.4	294.4	+ 76.5
	(*b*) Control. . . .	23	9.8		370.9	
2	(*a*) "Iodine" . .	11	12.1	+ 0.9	364.1	+ 10.9
	(*b*) Control. . . .	15	13.0		375.0	
3	(*a*) "Iodine" . .	8	8.5	+ 2.5	311.5	+ 67.1
	(*b*) Control. . . .	10	11.0		378.6	
4	(*a*) "Iodine" . .	6	12.0	− 2.7	251.7	+ 59.6
	(*b*) Control. . . .	6	9.3		311.3	

It is evident that neither in the case when iodine feeding was begun earlier (first and second experiment) nor in that started almost a week later (third and fourth experiment) the obtained results were concordant. The sign of the difference between the terms in control animals and those for the experimental materials was different in both cases which points to the lack of any influence of iodine feeding. On the other hand the average weight of the "iodine" chrysalids was here also always smaller than that of the control pupæ. The total of the recorded chrysalids amounted here to 37 "iodine" and 54 control specimens. My material was therefore larger than that of Gedroyć. It was nevertheless not large enough to consider the results obtained as decisive, all the more as the insects were not segregated in respect to sex. Further and more detailed research ought to be undertaken in this respect.

It ought to be remarked, that notwithstanding such distinct influence of both thyroid and Lugols' solution on growth of caterpillars, mortality in the animals of the experimental materials was in general not larger than in those of the control. The average mortality in male "thyroid" larvæ attains ca. 6 per cent., in females 5 per cent., in the control materials 4 per cent. in males and 7 per cent. in females. Analogous data for "thyroid" chrysalids was 7 and 3 per cent., in control pupæ 6 and 2 per cent. The larvæ of *Pieris brassicæ* reared as a rule very badly in the extremely damp air under glass, but also here no specially negative influence of the experiment could be observed. Viz. the average mortality in the "iodine" caterpillars was here approximately 43 per cent., in the control material 38 per cent.

TABLE V.

THE COËFFICIENTS OF CORRELATION BETWEEN THE WEIGHT OF CHRYSALIDS OF *Lymantria dispar* L. AND THE LENGTH OF THE LARVAL PERIOD.

$r \pm E_r$, coëfficient of correlation together with its probable error; r/E_r, ratio of the coëfficient to its probable error.

Material of Specimens.	Number Corresponding to Each Experiment.	Males.		Females.	
		$r \pm E_r$.	r/E_r.	$r \pm E_r$.	r/E_r.
"Thyroid"	1	− .425 ± .072	5.9	+ .095 ± .090	1.1
	2	− .161 ± .080	2.0	− .408 ± .081	5.0
	3	− .506 ± .071	7.1	− .207 ± .106	2.0
	4	− .652 ± .051	12.8	− .251 ± .091	2.8
Control	1	− .476 ± .078	6.1	+ .033 ± .084	0.4
	2	− .278 ± .082	3.4	+ .460 ± .069	6.7
	3	− .415 ± .089	4.7	− .130 ± .092	1.4
	4	− .606 ± .079	7.7	+ .279 ± .088	3.2

After the present experiments were finished an abstract appeared [1] referring to a recently published preliminary note of Terao and Wakamori ('24) inaccessible to me. The authors report that the metamorphosis of the caterpillars of *Bombyx*

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[1] *Berichte ü. d. ges. Pysiol. u. exp. Pharmak.*, Vol. 28, p. 119, November 12, 1924.

mori L. fed on mulberry leaves sprayed with thyroid extract is normal and that the "thyroid" animals are smaller than the controls. The accelerating influence of the thyroid food was however noticed in the second generation of the insects under examination. We see that the results of my "thyroid" experiments are in complete agreement with those performed by Terao and Wakamori on the first generation of the animals. Unfortunately I have been unable to examine the second generation of my moths.

I will not discuss here the relation of the lack of any influence of thyroid on the rate of metamorphosis of the insects experimented upon to the problem of the physiology of metamorphosis in these animals. As I have emphasized elsewhere (Kopeć, '23), only positive results of the experiments on the influence of glands of internal secretion may be considered as decisive in this respect and only to a certain degree. It is not impossible that the negative influence exerted in my experiments on the weight of caterpillars by thyroid feeding is not due to specific checking of growth faculties, but rather to certain changes elicited in their digestion by a totally foreign substance. Such caution is the more advisable, as we have already certain data in the literature, which point to positive effect exerted on "lower" animals by substances of glands of internal secretion from vertebrates. Nowikoff ('08) remarks an increase of the rate of multiplication of infusorians reared in aqueous extracts of the thyroid gland. Hankó ('12) observes a positive influence of extracts of hypophysis on the rate of moult, regeneration and growth in *Asellus aquaticus*. The thyroid gland has, according to Wulzen ('16) a positive influence on the rate of division and growth of *Planaria maculata*. From the experiments of van Herwerden ('23) we see that the cortical substance of adrenals of the ox has an accelerating influence on the multiplication of *Daphnia pulex* as well as on growth of this crustacean and of young specimens of *Limnæa stagnalis*. I believe however that only a larger experimental material may constitute a base for general discussion on the influence of glands of internal secretion of vertebrates on processes taking place in invertebrates.

The opinions of separate authors as to the relation of growth to metamorphosis are not as yet in agreement. While for certain authors metamorphosis is a function of growth—for others there is no essential connection between the two processes. My experiments furnish a new support for the latter opinion, if of course we shall agree to draw inferences as to growth from the weight of the animals. We see that in "thyroid" caterpillars notwithstanding a considerably decreased weight, the rate of metamorphosis remains totally unchanged. If metamorphosis were a function of growth, the correlation between the weight of the pupæ and the duration of larval life would be, at least in normal conditions, always negative. It is therefore very characteristic that both in the "thyroid" and in the normal material the coefficients of correlation between the weight of the pupæ (*i.e.*, the measure of growth) and the duration of the larval period were, it is true, in general rather large, but not always negative (cf. Table V.). According to my opinion we may infer therefrom that between larger growth and quicker metamorphosis of caterpillars there is only the relation of contemporariness, perhaps dependent on a totally separate, unknown factor (or factors). We have not to do here with real, functional correlation between growth and metamorphosis which could not be abolished even in normal conditions.

In conclusion I want to remark that the results of my present research are by no means contradictory to my former views on the decisive rôle of the brain for insect metamorphosis, during normal development (Kopeć, '22) as well as during starvation (Kopeć, '24). The substance of the thyroid gland from vertebrates may have no relation to the substance (or substances) the existence of which ought, according to my opinion, to be considered as indispensable for the metamorphosis of these animals. I refer their presence in the insect to the presence of brain. The lack of metamorphosis in the caterpillars of *Lymantria dispar* which have been deprived of brain the seventh day after their last moult (Kopeć, '22) is sufficient evidence in this connection. The only matter for discussion is whether this influence occurs through the interaction of nerves, or, according to my supposition (not assertion), by means of internal secretion. In recent times Gedroyć ('23) opposed very decisively my interpretation of the hormonal rôle of the brain during insect metamorphosis. Namely when grafting brains or other parts of the nervous system on normal caterpillars of *Deilephila euphorbiæ* this author did not observe any acceleration of metamorphosis. I cannot, however, consider as decisive experiments like those of Gedroyć. in which the influence of grafted parts of the nervous system are studied, the analogical parts of the host not being removed. The introduced nervous tissue is present here in excess and it *might* at most, but it *need* not elicit accelerated metamorphosis, *i.e.*, increase the effect of the function which brain probably has when present in the organism in normal quantities.

I want to explain here a certain seeming contradiction between the results of my former experiments, which has been justly pointed to by the mentioned author. Gedroyć emphasizes that from Table I. of my paper on the rôle of the brain in the metamorphosis of insects (cf. Kopeć, '22) it follows that the control caterpillars which fasted since the seventh day after their last moult, and the head of which has been injured, the brain being not removed undergo pupation simultaneously with the caterpillars which have not been operated upon at all, and which were not deprived of food. On the contrary from my later paper on the influence of starvation on the development of insects (cf. Kopeć, '24) it follows that caterpillars which have been subjected to inanition since the seventh day after their last moult undergo pupation later than the unstarved controls. This discrepancy is however not essential for the caterpillars subjected in either case to starvation the seventh day after their last moult were not physiologically identical. In the second case (Kopeć, '24) they were normally fasting animals, in the first (Kopeć, '22) injured similarly to those the brain of which has been removed. Owing to the severe injury, connected with loss of blood, these caterpillars were not able to take food and were therefore subjected indirectly to starvation. I have twice just emphasized in connection with other experiments (Kopeć, '08 and '11) that serious operations on caterpillars may have an accelerating influence on the succeeding moult, which in the above mentioned case corresponded to pupation. This accelerating influence on metamorphosis may thus have annulled in the case from my paper of 1922 the hindering influence of starvation. In other words, probably owing to mutual counteraction of the two opposite influences the normal term of pupation was maintained here.

From the foregoing inquiry the following summary may be given:

1. The administration of thyroid to caterpillars of *Lymantria dispar* L. did not cause any distinct changes in the duration of the larval nor of the pupal period. The only effect was a distinct diminution of the weight of the chrysalids.

2. Neither the variability of the pupation term, nor that of the emergence of the moths, nor of the pupal weight underwent any regular or essential changes as compared with corresponding items of the control materials.

3. Lugols' solution (IK + I) had no distinct influence on the rate of pupation of the caterpillars of *Pieris brassicæ* L. and its only consequence was the negative changes of pupal weight. In respect to the scanty material these experiments are however not considered as decisive.

4. Negative correlation between the growth of caterpillars of *Lymantria dispar* L. (the growth being expressed as weight of the pupæ) and the duration of larval life cannot always be observed. This fact as well as the diminution of growth in spite of the unaltered rate of metamorphosis in the "thyroid" experiments point to phenomena of concommitance but not of functional correlation normally existing between metamorphosis and growth of insects.

5. The mortality of the "thyroid" as well as of the "iodine" specimens was not larger than in the control materials.

6. The hypothesis formerly uttered by the author as to the hormonal influence exerted by the brain on insect metamorphosis is by no means abolished by the negative results of the present experiments. Neither is this hypothesis cancelled by the experiments and considerations of Gedroyć.

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SINGLE AND DOUBLE RINGS AT THE REDUCTION DIVISION IN UVULARIA.

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It has not, apparently, been yet ascertained in what points the reduction division in flowering plants differs from the corresponding division in animals, such as the insects (see Tischler, 1922). The absence of a centrosome, indeed, has been noted in many flowering plants; and it has also been presumed that the division of the homologous chromosomes into chromatids is not visible before the early anaphase. But whether the complicated changes described, for instance, by Janssens (1924) as occurring in insect chromosomes and summarized by Wilson (1925), are paralleled in flowering plants, is, it appears, not known (though Chodat, 1925, considers that *Allium* offers a parallel). The following is a small contribution to the determination of the likenesses and differences of the maturation divisions of flowering plants and those of the best known animals, say, the Orthoptera.

As compared with most animals, flowering plants may differ in showing a periodicity in the occurrence of the maturation divisions corresponding with the alternations of day and night. Certain stages of *Uvularia*, for example, apparently usually came late at night and hence were rarely obtained under normal environment in the daytime. However, cold checks or stops the process; and if preparations are made at intervals after cold has occured in the night, various less common stages can often be procured in the daytime. The first and second metaphases of *Uvularia* have been readily obtainable under the usual environment; but the stages just previous to the first metaphase, and those showing the separation of the bivalents into their component chromosomes, or chromatids, were not often seen when preparations were made at 9 or 10 o'clock in the morning. The preparations were mostly procured after the young buds had been somewhat chilled by the temporary lowering of the night temperature in February and March, when the greenhouse fires were banked.

The preparations of young microspores and pollen-mother-cells were fixed and stained in iron-acetocarmine (Belling, 1921). A water-immersion objective was used to examine the chromosomes. It may be noted that an apochromatic water-immersion of 1.25 aperture will allow a true condenser aperture of 1.2, if the source of light is diaphragmed to fit the field of view. The working aperture of the combination is about 1.2. On the other hand, the working aperture of the 1.4 N.A. apochromatic oil-immersion, used with a dry Abbe uncorrected condenser on objects in iron-acetocarmine, is only 0.9 or less. (Compare Belling, 1923.)

Three hypotheses have been made as to the rings seen at the metaphase in certain Liliaceæ and other genera of flowering plants. (1) That these rings result from the mere twisting of the constituent chromosomes of the bivalents around one another, and that these become untwisted and longitudinally split at the anaphase. This hypothesis is, it would seem, negatived by the fact that, with the best visibility in the microscope, it is usually impossible to say which chromosome is above and which below at the junctions. (2) That the rings are caused by alternate openings between the homologues and between their constituent chromatids, so that adjacent rings are always at right angles. This hypothesis does not appear to agree well with the state of affairs in the trivalents of the triploid hyacinths, where 3 chromosomes are concerned (Belling, 1925*a*). (3) That between each two adjacent rings there is a chiasma in Janssens' sense (or a similar interlacing of strands) where segmental exchange may have taken place, by the fracture and subsequent reciprocal junction of two of the four strands. This assumption seems most suitable for a working hypothesis, since crossing-over (and hence probably segmental interchange) has been shown to occur in those flowering plants which have been investigated *ad hoc*.

However, in the present paper only the following points are regarded.

1. Whether in horizontal rings or V's one chromatid passes up and one down from each lateral half of the ring or V.

2. Whether these chromatids in separating show signs of inter-

lacing at the junction, so that a ring or V gradually diminishes in size as its chromatids are pulled into the loop by the spindle fibers.

3. Whether, in vertical rings or V's, the process is the same as the above; or whether the upper and lower halves of the rings or V's separate as wholes.

4. What different configurations are shown by the same homologous chromosomes in different cells.

Bivalent I.

Chromosome I., in the pollen grain (Fig. 1), is seen to be formed of two slightly unequal segments, and is usually bent; the spindle fiber being, of course, attached at the constriction, where the two segments are connected by a fine thread. Fig. 2 of the paper on the origin of chromosomal mutations (Belling, 1925*b*) shows bivalent I. in the late prophase in the form of two medium-sized

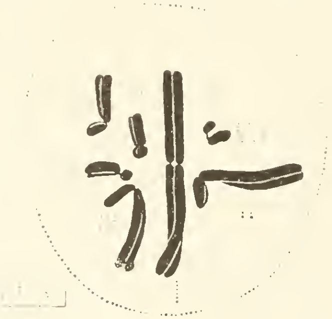

FIG. 1. This shows the metaphase at the first division in the pollen-grain. (Like all the other figures it was made from preparations of *Uvularia grandiflora*.) This pollen-grain was selected from a large number as showing the chromosomes well spaced. The longitudinal divisions and the constrictions are evident. It can be seen that the two segments of chromosome I. are of unequal length. Chromosome III. has apparently a shorter long segment and a longer short segment than chromosome II. The small segment of chromosome V. is closely attached to the large segment, while in chromosome VI., the two segments are separated by a thread.

This, and the subsequent figures were drawn with the camera from iron-acetocarmine preparations, the light being screened by Wratten yellow-green filter No. 56. A Leitz' achromatic aplanatic condenser, corrected for use as a water-immersion, was used to give a large cone of light. Zeiss' apochromatic water-immersion 70 was employed in all cases.

rings with a long V, the plane of the V being at right angles to that of the adjacent ring. At the metaphase, as shown in Fig. 2 of the present paper, one large ring and V may be present. The division into chromatids cannot be seen in face view, but only when the rings or V's are presented edgeways. In the next stage, which is not figured here, a small loop arises from the

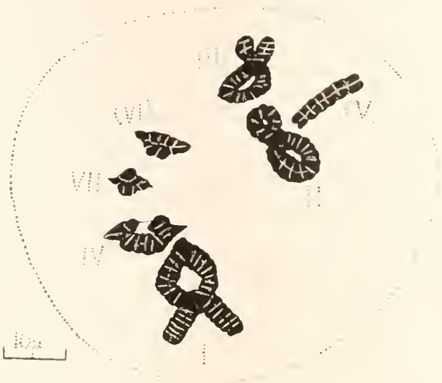

FIG. 2. First metaphase (to anaphase) in a pollen-mother-cell, from a plant forced in the greenhouse, in March, 1923. This cell was selected as showing no overlapping bivalents. In bivalents I., II. and III., the spindle fibers would probably be attached where the constituent chromosomes seem to cross.

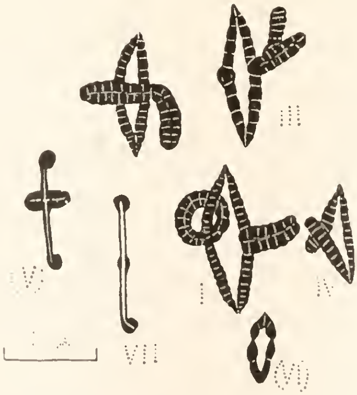

FIG. 3. First metaphase to anaphase in a pollen-mother-cell. Drawn in March, 1924; after a spell of cold weather. The drawing surface was shifted after outlining each bivalent, as was done also in Figs. 4 and 5. Bivalent VI. has the more or less exceptional configuration noted in the text in bivalent IV. Bivalent VII. is the most advanced in separation.

apposed points of constriction of the homologues, and is pulled out by the spindle fibers towards each pole, pulling the chromatids out of the rings or V's as it apparently increases in size at the expense of these rings or V's. In Fig. 3, bivalent I. consists of a large loop with two medium-sized rings; one of which is seen edgeways. This ring shows the division into chromatids. This bivalent perhaps started as two rings. In Fig. 5, lower line, bivalent I. consists of a diminishing ring and a V, with a large loop between them, formed probably at their expense. Bivalent I., in the upper line of this figure, is apparently similar but the ring is somewhat bent. In Fig. 3, of the paper on the origin of mutations, bivalent I. has the form of a large loop with two small rings, probably remnants of original large rings. In Fig. 4 of the present paper, upper line, bivalent I. has a ring on one side of the loop, while on the other side the ring may perhaps have been vertical, and the two halves have separated vertically. In the lower line of this figure, the halves of bivalent I. have separated at one end, but the last remnant of the V remains at the center. Finally, in Fig. 6, the 4 chromatids of bivalent I. are

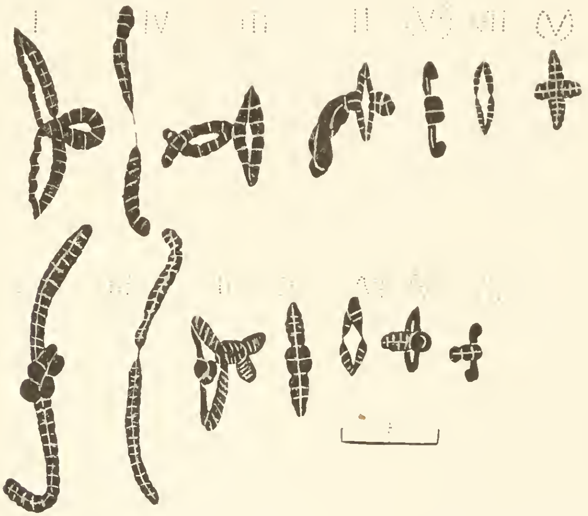

FIG. 4. Bivalents from two more selected cells showing the metaphase to anaphase, as in Fig. 3. Two bivalents, VI. and VII., are of the exceptional form.

clearly visible; and it may be that there had been a horizontal ring on the right, and a vertical ring on the left.

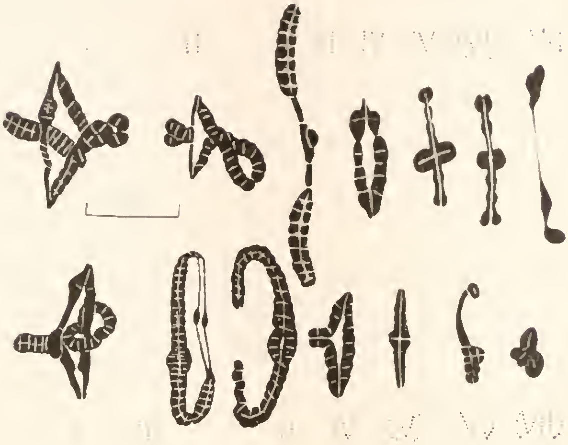

FIG. 5. Two more selected metaphase to anaphase groups. The last two bivalents in the lower line seem more or less malformed.

BIVALENTS II AND III.

Chromosomes II. and III. (Fig. 1) doubtless differ slightly in size, but are similar in behavior, and may be considered together. They are long J chromosomes, with a large and a small segment close together. At the late prophase (Fig. 2, Belling, 1925*b*) these two bivalents may have each the form of a large horizontal ring with a small V at one end, and a vertical portion consisting of the two small segments at the other end. At the metaphase (in Fig. 2, of the present paper), one of these bivalents forms a ring with a small V, and the other a large and small ring in different planes. Fig. 4 of the paper on the origin of mutations shows bivalent II. forming a horizontal ring with a small vertical piece consisting of the two small segments, and with a small loop arising from the constrictions. In the upper line of Fig. 4 of the present paper, this loop has increased in size in bivalent II. In Fig. 4, lower line, and Fig. 5, upper line, bivalent II. shows part of the small segments still in the equatorial plane, as well as a ring (or ring and V) on the other side. But in bivalents II. and III. of Fig. 3, the small segments have been quite drawn into the loop, and only the diminishing V is left; or in bivalent III. of the upper line of Fig. 4, the ring and V. In Fig. 5, lower line, both short and long segments have been drawn into the loop. Fig. 6 is especially instructive, for bivalent II. probably had consisted of a large horizontal ring with a vertical piece formed of the two short segments. This is seen to have separated by the horizontal splitting of the ring into its constituent chromatids, the small segments having first separated. In bivalent III. of Fig. 6, a horizontal V has, it seems, separated into chromatids in the same way.

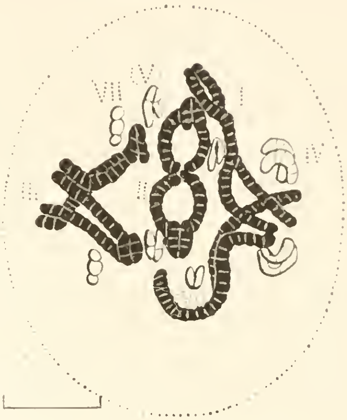

FIG. 6. A rare stage of the pollen-mother-cell at the early anaphase, showing the final separation of bivalents I., II. and III. The arrangement of the chromatids of bivalent II. must apparently be due to the horizontal splitting of a horizontal ring. The four smaller bivalents are, as usual, ahead of the larger ones in completing the separation of their constituents.

BIVALENT IV.

Chromosome IV., as shown in Fig. 1, consists of a small J, in which the two segments are close together. It is distinctly larger than either V. or VI. In Fig. 2 of the paper on the origin of mutations, bivalent IV. is formed of a horizontal V, with a vertical piece consisting of the two short segments. In Fig. 2 of the present paper, this bivalent, which apparently started as a ring, is already in the early anaphase (for the small bivalents usually separate before the large ones). In Fig. 3, bivalent IV., which possibly started as a ring and V, still shows a small V at the left of the loop. A later stage is shown in the lower line of Fig. 5. In the top of Fig. 4, the two halves of bivalent IV. have separated without splitting lengthways. The constriction between the short and long segments is visible. The upper line of Fig. 5 shows a different configuration for bivalent IV., in which the two sides of the loop are equal. This is intelligible, first if we suppose the point of attachment of the spindle fiber and the constriction to have been shifted to the center of both chromosomes, which seems unlikely. Or we may presume that a horizontal V has separated into its chromatids, somewhat as bivalent III. of Fig. 6.

Bivalents V. and VI.

Chromosomes V. and VI., since they differ only in VI. having its small segment attached by a long filament, cannot be told apart in the maturation divisions, and must therefore, be considered together. In Fig. 2 of the paper on the origin of mutations, bivalents V. and VI. have the form of V's or truncated A's. In Fig. 3 of the present paper, in the configuration marked (V.), the two small segments have separated, and the two large segments are still horizontal and show the split between the chromatids. Similar forms may be seen in the top line of Fig. 5. The bivalent marked (VI.) in Fig. 3, however, like the one in the bottom line of Fig. 4, is presumed to have come from the separation of the chromatids of a horizontal V.

Bivalent VII.

Finally bivalent VII. resembles V. and VI. in its configurations, but is recognizably smaller.

To sum up: (1) There is, apparently, fusion at the junctions of the two constituents of each bivalent. (2) Horizontal rings and V's divide into their constituent chromatids along a horizontal plane, the line of division being distinct at the metaphase. (3) Vertical V's and rings have not been observed in the act of separating; but they probably separate into upper and lower halves. (4) The rings and V's apparently diminish as the loop between them is pulled out by the spindle fibers. This would presuppose crossing or interlacing of the chromatids.

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BIOLOGICAL BULLETIN

ON THE PHOTOGENIC ORGAN OF THE KNIGHT–FISH (*MONOCENTRIS JAPONICUS* (HOUTTUYN)).

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It was Stead (1906, p. 89) who first described the luminescence of the knight-fish (*Monocentris gloria-maris*). "On each side of the head near the mouth are peculiar luminous discs, which are probably of service to the fish in assisting it to obtain its food." Unfortunately this interesting observation has escaped attention up to this date.

Monocentris japonicus, another well-known species of the knight-fish, is a rather common animal along the coast of Japan. This fish also has the same property of light production as the Australian form. Light is produced by a pair of glands situated just under the lower jaw of the fish. Their function and structure were described by Yoshizawa (1916, p. 411) in Dobutsu-Gaku-Zashi, a Japanese zoölogical periodical. His brief description was illustrated with figures, but several points remain unexplained. According to Yoshizawa the photogenic organs of *Monocentris* have the construction of photospheres, composed of large glandular cells with nuclei of a considerable size, but without accessory structures such as the reflector. He seems not to have been aware of the truly glandular nature of the organs.

In 1917 Harvey came to Japan and made at the Misaki Biological Station some observations upon the interesting luminous shore-fish. These observations have been recorded in several of his papers. The photogenic property of *Monocentris* thus has come to be generally known as a fact.

LOCALIZATION AND GENERAL STRUCTURE OF THE PHOTOGENIC ORGANS.

The photogenic organs of *Monocentris* consist of two ovoid protuberances lying side by side on each side of the median thickening of the lower jaw, their longer diameter being nearly at right angles to the median plane (Fig. 1, *gl*). In a fish 12 cm. long the protuberances measure about 4 mm. in length and 3 mm. in width. Their whole surface being covered with minute dermal papillæ of dark brown color, the photogenic protuberances do not stand out clearly above the surrounding surfaces. This fact seems to be the reason why the organs in question have remained unnoticed by most ichthyologists.

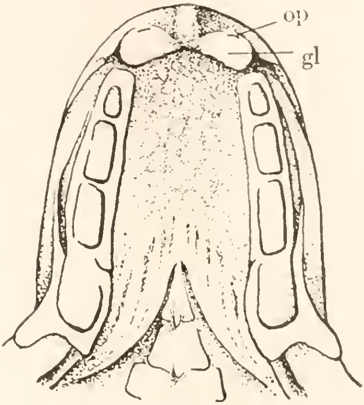


FIG. 1. Ventral view of the lower jaw of *Monocentris japonicus*. About × 2. *gl*, photogenic gland; *op*, opening of gland.

The photogenic organs of *Monocentris* are glands of an excretory nature, although luminous material does not escape under normal conditions. The glandular nature of the organs can easily be demonstrated even without dissection. Each photogenic protuberance has a slit-like opening at the anterior end (Fig. 1, *op*) and when pressed under conditions of darkness a luminous fluid is observed to be forced out through this point. The opening is crescentic and its longer diameter is almost parallel to that of the protuberance.

The interior of the photogenic organs is white and spongy, containing a large quantity of minute crystals. A great number of simple tubules develop around the central tissue.

They crowd so thickly that each stands almost vertically, and they lie parallel to one another. Indeed, the tubules appear to belong to a single gland with several large central spaces (Fig. 3, pl. I.).

Each photogenic organ of *Monocentris* is, however, a compound gland made up of some nine components which unite secondarily into a functional whole. The distal part of the gland still shows the compound nature and this part consists of many narrow canals, each representing the duct of an individual acinous gland. The general constitution of the organ is represented in Fig. 2.

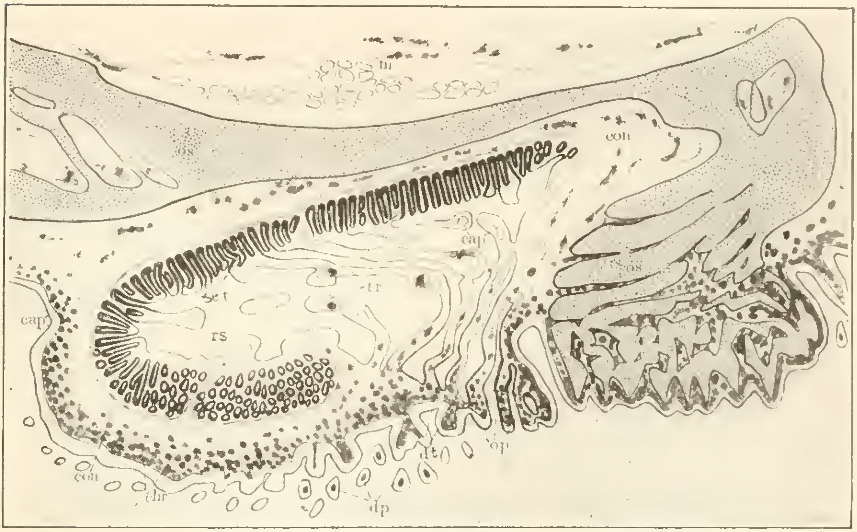


FIG. 2. Sub-mental photogenic gland of *Monocentris japonicus* in longitudinal section passing through its opening. About × 30. *cap*, capillaries; *chr*, chromatophores; *con*, connective tissue; *dp*, dermal papillæ; *dt*, emissory ducts; *m*, muscles; *rt*, reservoir; *op*, openings of gland; *os*, sub-maxillary bone; *se. t*, secretory tubules; *tr*, trabeculæ.

HISTOLOGY OF THE PHOTOGENIC ORGANS.

Under a high magnification each tubule shows an epithelial lining composed of cubical cells arranged in a single layer except at the fundus region which is the seat of a most active secretion, where the walls are considerably thickened with two or three cell layers (see Fig. 4, pl. I.). The stratified aspect of this region is, however, not due to the direction of cutting but is the result of actual proliferation of epithelial cells. Toward the exterior at a certain distance from the fundus the stratified aspect is no more to be noticed, the cells being generally lower and the secretion less in amount. Thus, in transverse sections of the tubule, the fundus and distal region show a structural difference, the walls of the former being thick and stratified, while those of the latter are thin and simple. Naturally the difference is a gradual one. The caliber and length of the tubules are also variable, but the largest ones are generally situated at the proximal region of the gland.

A certain quantity of secretion is always found in the tubules. It is granular in composition and readily stainable with hæmatoxylin. The epithelium of the tubule is completely destroyed in the process of secretion instead of repeating its function a number of times as ordinary secretory epithelium does. Each cell of the epithelium, in this case, does not function independently but operates in connection with others along the considerable extent of the walls of the tubule. The new epithelium is replaced by the constant proliferation of cells. The process is especially active at the fundus region of the tubule, and here the process is so active that more than one layer is produced before the uppermost one is entirely destroyed. This fact gives rise naturally to the stratified aspect of the epithelium at the fundus region. The nuclei of these cells are ovoid and contain the chromatic substance in normal amount. They do not deteriorate in a degree visible to the eye as far as the cellular destruction occurs. By the time when the cell body is completely disintegrated and the nuclei are set free into the lumen of the tubule, the latter lose their internal structure, their chromatic substance being reduced in amount. Yet the nuclear membrane remains unaltered and shows, adhering to its inner surface, the last scattered traces of the chromatic substance (Fig. 5, *n*′, pl. I.).

As has been stated, the secretion is of granular constitution. Whether or not the granules are preëxistent in the cells is difficult to say. They have been described in the cells of *Anomalops* and *Photoblephalon*, two luminous shallow-water fishes in the

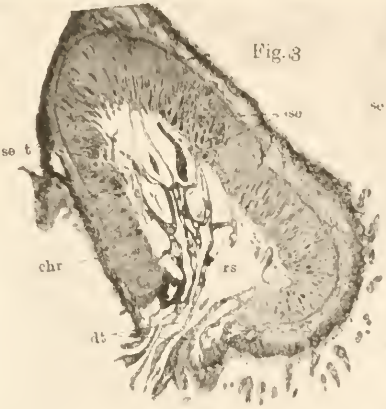


Fig. 3

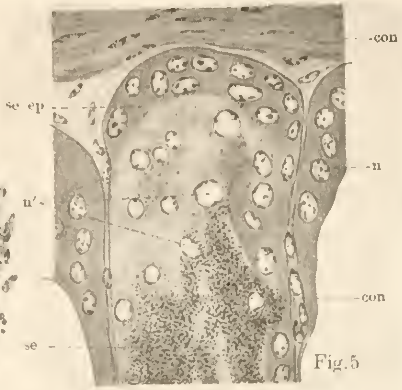
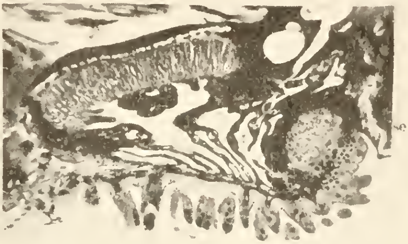


Fig. 5

Fig. 6



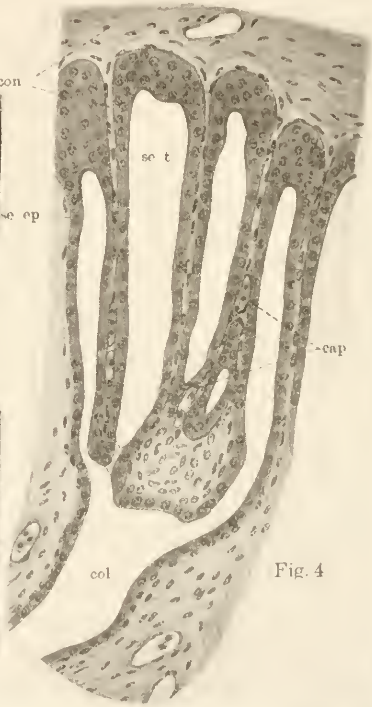


Fig. 4

Fig. 7

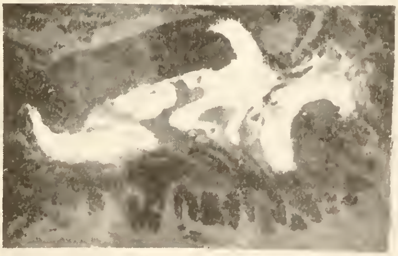


FIG. 3. Sub-mental photogenic gland of *Monocentris japonicus* in horizontal section at the level of its opening. × 16. *chr*, pigment layer; *dt*, emissory ducts; *rs*, reservoir; *se*, secretion; *se t*, secretory tubules.

FIG. 4. A portion of gland proper. × 640. *cap*, capillaries; *col*, collecting tubule; *con*, connective tissue; *se ep*, secretory epithelium; *se t*, secretory tubule.

FIG. 5. Basal part of a secretory tubule. × 1,250. *con*, connective tissue; *n*, nuclei in epithelium; *n'*, nuclei in secretion; *se*, secretion; *se. ep*, secretory epithelium.

FIG. 6. Sub-mental photogenic gland of *Monocentris japonicus* viewed by transmitted light. × 16.

FIG. 7. The same gland seen by reflected light. Crystalline deposit is represented in white.

Malayan Sea, and the photogenic organs of these fishes closely resemble those of *Monocentris* (see Steche, 1909, p. 349). No granules have, however, been detected in the cells of the latter. I am rather of the opinion that the granules are produced through the metamorphosis of the cytoplasm at the time of cellular destruction as Förster (1914) has described in *Pholas dactylus*. On the other hand, Harvey (1921, p. 43) claims the granular bodies in the tubules to be symbiotic bacteria which cause the luminescence. To this problem I shall return on another occasion.

The secretion produced in the way just considered is carried from the tubules toward the medullary part of the organ through a number of tubules of the second order, or the "collecting tubules" (Fig. 4, *col*). The latter proceed more or less parallel to one another but separated by irregular trabeculæ of connective tissue. They communicate often in their course and finally at the centre of the organ constitute several large lumina, or the "reservoirs" (Figs. 2 and 3, *rs*), in which the secretion is stored and probably oxydized with light production. The reservoirs communicate also with the outer medium by a number of long, well-defined, parallel tubes, or the "emissory ducts" (Figs. 2 and 3, *dt*). About nine such ducts are generally observed.

The inner walls of the collecting tubules and reservoirs are lined by a low, simple epithelium, while those of the emissory ducts are covered by one which is stratified, a direct continuation of the epidermis of the surface.

Beyond the tubular area—the gland proper—the photogenic organ is enclosed externally by a thick dermal layer in which a great number of chromatophores are found. The latter are especially abundant on the outer surface of the organ. However, they are scattered and do not constitute a pigment cap (Figs. 2 and 3, *chr*).

The blood is supplied by branches of the lower jaw arteries which run near the organ. They enter into the gland proper, passing through the surrounding dermal tissue, and on reaching the tubular area branch out all at once into many capillaries, each making its way between the secretory tubules. After passing the tubular region the capillaries gather themselves again to form veins which enter into the connective-tissue trabeculæ of the medullary part of the organ.

The tissue of the central part, and to some extent that which encloses the proximal part of the organ, is always loaded with a large quantity of very minute crystals which appear chalky white by reflected light and brown by transmitted light (comp. Figs. 6 and 7, pl. I.).

Harvey states that the luminescence of *Monocentris* is continuous. The fish can produce light both day and night. But the emission of light seems to be controlled to some extent by internal stimuli. The operating mechanism has not, however, been completely ascertained. No special muscle fibers have been found in the organ. Nevertheless it may not be unjustifiable to suppose that the thin stratum of more or less elastic fibers surrounding the outer surface of the tubular region exerts a contraction by which the spontaneous light is produced. Otherwise it is quite difficult to understand the spontaneous luminescence occasionally produced by the fish. This stratum is not clearly differentiated from the ordinary dermal tissue.

I have not been able to detect any nerves entering the gland proper.

Observations on the Living Fish.

In connection with this morphological study on the photogenic organs of *Monocentris* I have made some biological observations on the living fish. The emission of light was seen in darkness under certain conditions. It could easily be produced by agitating the water in which the fish was kept or by adding to it a few drops of ammonia, but very often the luminescence was spontaneous. Luminescence is continuous for even several hours. By rubbing the luminous protuberances with a piece of stick or by scraping them with a knife edge, the luminosity could partially be transferred to the surface of the stick or knife, remaining visible there for several seconds. Although it will be seen that in this case we are dealing with a substance excreted by the organs, it should not be considered that the luminescence is ordinarily due to excreted matter—the "external luminescence"—because no luminous material was seen excreted into the water by a living fish; the luminescence is "extracellular" but "intraglandular."

Monocentris can live in fresh water for a considerable length of time, even for ten hours. Nevertheless, they are quite uneasy under such conditions, as indicated by rapid beats of the fins, stirring movement of the tail, a bubble formation on the skin and, especially, the emission of a continuous bright light.

Monocentris can live in the sea water within a range of temperature between 0° C. and 40° C., and light is produced within somewhat wider limits, the maximum temperature being about 42° C. and the minimum about − 10° C.

Monocentris can show light at any time of the day and night, but the natural luminescence seems to cease in the day time, for the fish after being removed from daylight to darkness did not show this property at least for ten to fifteen seconds.

The regeneration experiment of the photogenic organs, which I have performed, may be interesting and is somewhat suggestive in regard to the problem of the animal luminescence in general. The photogenic organs of eight fishes were excised in varying extent. The wound healed very soon and in the course of about two weeks the integument was completely regenerated. The internal changes following these operations were studied by means of serial sections. The result shows that the tubules of the organs, either completely or partially removed, are not regenerated but are replaced by a spongy tissue consisting of exceedingly enlarged blood vessels. If the granules in the organ were bacterial bodies, they might have been expected to increase in amount, because they were subjected to a favorable condition with an abundant supply of blood. But this does not seem to have been the case. There was no increase in the amount of granular matter in the remaining tubules.

Similar Photogenic Organs of Other Fishes.

Finally the only photogenic organs which at all approach those which have been considered are to be found in two Malayan fishes, *Anomalops katoptron* (Bleeker) and *Photoblepharon palpebratus* (Boddaert) which have been made the subject of memoirs by Steche (1907, p. 85 and 1909, p. 349). A glance at his figures in Pls. XIX–XXI will at once show the fundamental similarity of the "sub-ocular" organs of the *Anomalopidæ* and the "submental" organs of the *Monocentridæ*. However, the reservoirs and emissory ducts which are well developed in *Monocentris* are wanting in both *Anomalopsis* and *Photoblephalon*. In the latter all of the several secretory tubules communicate with the external medium by one very short common duct. But each photogenic organ in question is an aggregation of many acinous glands developed in a limited area sub-ocular in position, and the secretory tubules stand vertically and lie parallel side by side as in the case of *Monocentris*.

The crystalline deposit is also present. The chromatophores are described as forming a pigment cap which encircles the proximal surface of the organ.

In *Anomalopsis* the oral end of the photogenic organ is said to be fastened to a long cartilagous shaft, upon which it is rotated downwards so as to bring the luminous surface against the body wall and thus cut off the light. In *Photoblephalon* the action of shutting off the light is done by a moveable screen. No analogous mechanisms of light regulation have been found in the submental photogenic organ of *Monocentris*.

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THE DEPENDENCE OF SECONDARY SEX-CHARACTERS UPON TESTICULAR HORMONES IN LEBISTES RETICULATUS.

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I. INTRODUCTION.

The problem of sex as a chapter in the mechanics of development has not been investigated until lately. A number of authors whose investigations were performed on different animals have proven that those sex-characters which are generally called secondary sex-characters depend in their development, on hormones produced in the sex glands. After castration these characters will disappear. Proof to this effect has been produced by Steinach, Sand and Lipschütz in mammals; Pezard, Goodale and Zawadawsky in birds; Harms, Meisenhaimer, Witschi, Bresca and others in amphibians.

The dependence of secondary sex-characters in fishes was not at all investigated until quite recently. The reason for this may be found in the fact that those species which are endowed with permanent dimorphism, the exotic inhabitants of the aquaria, are distinguished by a very scant size and want of stability and therefore, are almost inaccessible for operative intervention.

As to seasonal dimorphism we find in the works of Courrier (2) and Von Oordt (5, 6, and 7) on the stickleback (*Gasterosteus pungitius* and *G. aculeatus*) indirect indications of its dependence on the hormones of the testis.

A direct experiment was carried out by St. Kopeć (4) who succeeded in castrating *Phoxinus lævis* males and females and keeping them alive for three weeks after operation. During this time said fish failed to exhibit nuptial colors at the usual time.

The hybridological analysis of the sex-characters in fishes was carried out by Aida (1) on *Haplochihis latipes* and by Winge (8 and 9) on *Lebistes reticulatus*. Both authors came to the conclusion that the color characters of the male depend on the genes located in the sex-chromosome, especially in the Y-chromosome.

The *Lebistes* female, even if its X-chromosome contains genes of color characters, does not phenotypically exhibit the latter.

II. Description of Material and Methods.

In connection with genetic researches on *Lebistis reticulatus* I had the opportunity to observe a great many of those little fishes in the Moscow aquaria. Among the members of this species as well as among members of other species which have come under my observation I very seldom met with divergencies from usual dimorphisms.

My material consists of seven fishes, six of which are males with marked divergence from typical dimorphism. The seventh is a case of hermaphroditismus glandularis. The malformation of dimorphisms of the males were either inborn or developed later in the life of the individuals. Two males, No. 25 and No. 34, belong to the former class.

1. *Male Fish No. 25.*—The individual measured 37 mm. in length. It was entirely deprived of dimorphic colorations. The shape of the body resembled that of a female. Upon dissection, after the fish died, it was discovered that the testis was entirely atrophied.

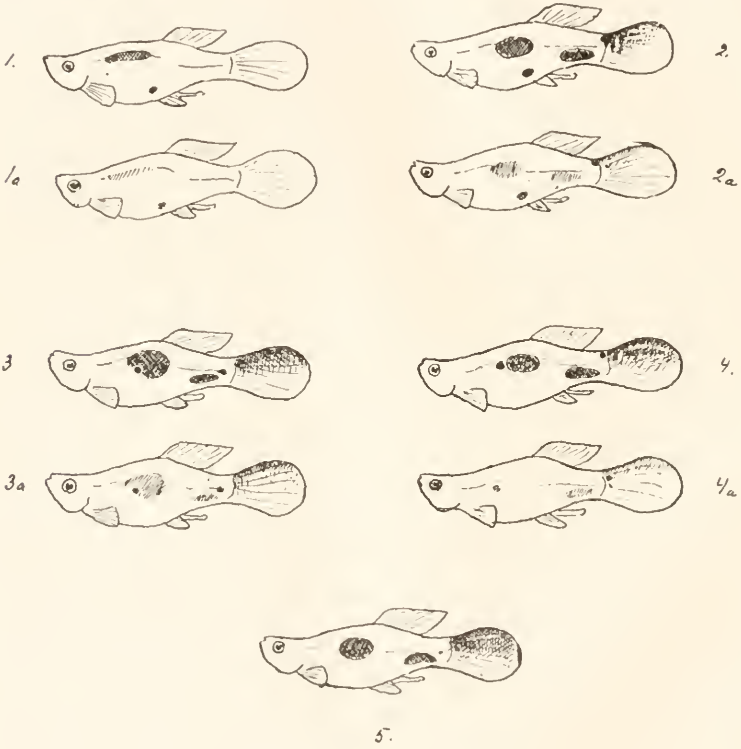
2. *Male Fish No. 34.*—It was born on the 5th of August, 1924. On the 5th of May, 1925, when the animal was nine months old, it measured 23 mm. in length but failed to exhibit any sex-colorations. The gonopod was normally developed. Sex instincts were not lacking; repeated mating to fecundate females, however, proved a failure. No sign of a gonad was discovered macroscopically.

3. *Male Fish No. 23.*—This animal reached maturity in the first part of September, 1924. Up to February of the following year it remained quite normal as to dimorphic colors and sex-instincts. During February the characteristic red spot on the side of the body disappeared and the fish became almost colorless. It died on the 27th of May, 1925, and on dissection revealed entirely atrophied gonads (Figs. 1 and 1*a*).

4. *Male Fish No. 8.*—With normal dimorphism this animal reached maturity in August, 1924. Paleness of sex-colors was noticed in March, 1925. On April of the same year both of the

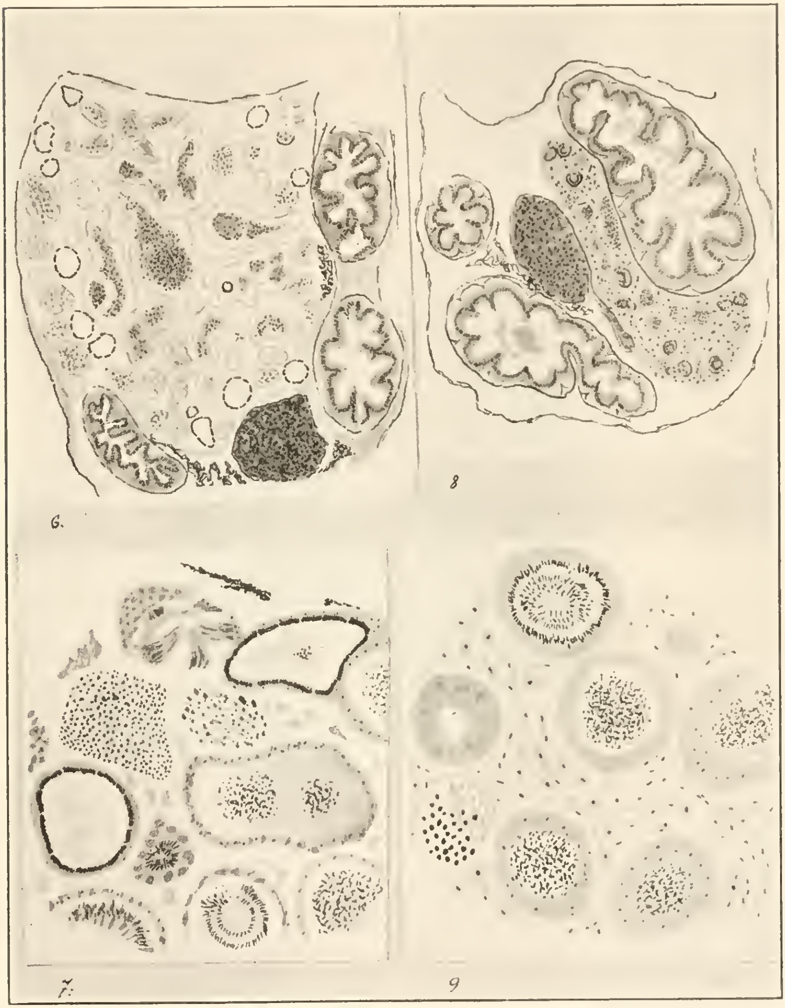
red spots were almost invisible. Also the black spots found on the side of the body lost their intensity. The specimen died on May 24, 1925. A very slightly developed testis was found after examination of the viscera. (Figs. 2 and 2*a*.)

5. *Male Fish No. 33.*—The specimen was born in August, 1924, and reached maturity with secondary sex-characters well formed. From May, 1925, on, the characteristic male shape was undergoing a change toward that of the female. This was closely followed by the disappearance of sex colorations. Figs. 3 and 3*a*. On June 4, 1925, it was killed and preserved in formol-alcohol solution. Sections eight microns thick were cut and stained in hematoxylin. Upon examination it was found that the testis was very small and almost deprived of spermatocysts. It contained neither primordial germ cells nor spermatocytes. Spermatogenesis was encountered here and there but only the later stages. The connective tissue of the gonad was well developed.



Figs. 1–5.

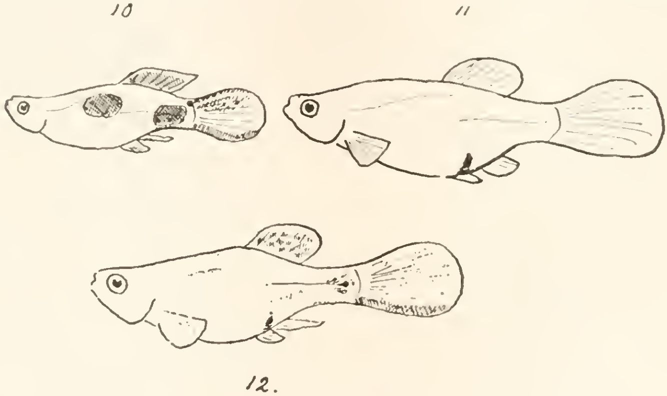
The coincidence of these two facts—loss of dimorphic colors and the atrophy of the testis—appeared significant and stimulated me to a more detailed study of the last "pale" male No. 44, Figs. 4 and 4*a*, parallel with its quite normal brother No. 47, Fig. 5.



Figs. 6–9.

6. *Male Fish No. 44.*—Phenotypically both fishes, No. 44 and No. 47 were very much alike. Both had two red spots in each

side with the upper part of the tail fin colored yellowish-orange. Male No. 44 had three black spots on each side while No. 47 possessed only one on each side. They were born on the 18th of September, 1924. Both fish developed and remained alike until May, 1925, from which time on male No. 44 gradually lost its coloration and on June of the same year was, so to speak, colorless (Fig. 4*a*). Both males were killed and fixed in formol-alcohol, sections made 6–8 micron in thickness and stained with hematoxylin. The histological picture of the normal male No. 47 showed a large testis and not less than 70 spermatocysts can be counted on one section. The connective tissue is poorly developed (Figs. 6 and 7). In case of male No. 44, the testes is only one fourth as large as that of its normal brother, only 16–17 spermatocysts can be found in one section with spermatogenesis almost reduced to nil. The connective tissue however is very well developed (Figs. 8 and 9). In the middle of the gonad of male No. 44 are found two cysts filled with an almost homogeneous mass. These cysts appear to be much like ova in process of degeneration.



FIGS. 10–12.

7. *Fish No. 59. The Hemaphrodite*—For this specimen I am very much indebted to Mr. N. J. Dragomiroff, who sent it to me from the University of Kiew. This fish had died 6 months of age and reached me preserved in Tellyesniczky's fluid.

The body of the *Libistes* hermaphrodite measures 37 mm. in length, which is a female characteristic, for the males never exceed

25 mm. The dorsal fin is rounded as is the case in normal females Near the anal orifice a dark black spot can be seen which characterizes the mature female in the viviparous fishes and which has been called "puberty spot" by Essenberg (3). The anal fin however has been modified towards the male direction and resembles a gonopod of not fully developed males, (Fig. 12). The colorations are more like those of the male than those of a female. The black spot found in the tail region is present in the hermaphrodite as well as the orange line on the lower margin of the tail fin. Compare Fig. 10, which is a normal female, Fig. 11, a normal male, and Fig. 12, the hermaphrodite. On dissection a fully developed ovary containing almost completely formed embryos was discovered.

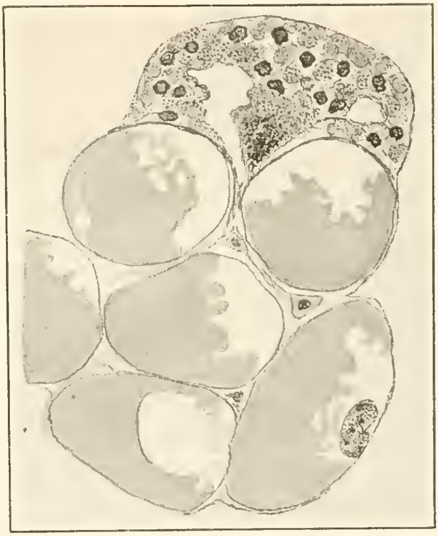


FIG. 13.

The gonad was embedded and sections, 8 microns in thickness, made. Material was stained in Heidenhain's iron-hematoxylin and Mallory's connective tissue stain.

The histological picture corroborates all the features that were observed by the unaided eye, that is, a well-developed ovary with embryos almost ready to be born. Besides all along the ovary, gradually tapering, was found a well developed testicle (Fig. 13).

Contiguous with the testicular tissue lies a mass containing disintegrated ovarian tissue (compare with Essenberg, 1923, Table 5, Fig. 25). In the testicular portion the various stages of spermatogenesis can be found. To all appearance normal and mature sperm cells gathered in cysts (Spermatophores) are just as abundant and regular as can be observed in normal testis.

DISCUSSION AND SUMMARY.

It was observed in the description of the six *Lebistes* males that the atrophy of the testis is paralleled by the disappearance of the male sex colors. Based upon these facts the writer concludes that the intensiveness, shape and development of the black and especially the red and yellow pigment spots depend upon the hormones produced in the testis in Lebistes.

With the disappearance of the male sex coloration the individual approaches the colorations of the female, that is to say, the female coloration is the characteristic coloration of the asexual forms.

In the case of mammals, birds and amphibians it is known that the coloration of the asexual forms approaches the sex colorations of that sex which is homozygos as to sex chromosome composition. In *Lebistes*, therefore, the male is heterozygos (XY) as to sex chromosome composition and the female is homozygos (XX).

Winge (8 and 9) has found in *Lebistes reticulatus* the presence of the gene complex S which is located in the X-chromosome and which is transmitted sex-linked. In other species of *Lebistes* (comprising the majority of the species of this genus), the X-chromosome is empty, that is devoid of color factors, and all color characters are transmitted by the Y-chromosome (one sided). The phenotypical manifestation of the S-complex in the males described above consists in the yellowish tinge of the dorsal fin and the orange line along the lower border of the tail fin and possibly the contour of the red spot on the tail.

No doubt the genes of the color pattern of the hermaphrodite belong to the S-complex. These are the orange line along the lower edge of the tail fin, and the red pigment on the tail Any other color characters the genes of which are located in the Y-chromosome, could not be found. Winge states that the Y-chromosome never is devoid of color factors. If that be so, our hermaphrodite lacks the Y-chromosome and from a genetic point of view it is a female.

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THE RÔLE OF THE NUCLEUS IN THE CELL FUNCTIONS OF AMŒBÆ.

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The importance of the cell nucleus in chemical synthesis of cell elements, regeneration, cell division, fertilization, and heredity is now appreciated. There are, however, many problems connected with rôle of the nucleus in the ordinary, everyday life of the cell which have not been so fully worked out. To many of these problems the metazoan cell forbids an approach because it is too differentiated, too specialized. In addition, it is difficult to eliminate the factor of interrelationship of cells in making such studies on the rôle of the nucleus. For this and other reasons the protozoa have supplied favorite objects for such studies. Because they lead a more or less autonomous existence they exhibit their vitality in a greater diversity of activities than do metazoan cells.

The researches of Gruber, Balbiani, Verworn, Morgan, Hofer, Stolć and many others have supplied ample evidence that an enucleated protozoan is incapable of regenerating any lost cell structure; *e.g.*, Morgan found that as much as 1/64 of a protozoan, *Stentor*, will produce a new individual if it contains a nucleus, but not if one is absent. The non-nucleated part will heal the wound, but will not regenerate lost cell organelles. Verworn too found that if he bisected a Foraminiferan, *Polystomella*, the part without the nucleus would not regenerate the material for a new shell as the nucleated portion did. An apparent exception is the contractile vacuole of amœbæ which, it is generally agreed, always appears anew in an enucleated fragment of an amœba not possessing one at the time of merotomy.

In ciliates generally locomotion is not affected by removal of the nucleus. There are, however, certain factors bearing upon the locomotion, irritability and nutrition of the cell, especially in the amœbæ, concerning which there is much disagreement. The method generally used in studying the bearing of the nucleus upon any phase of cell activity is by removing it, and observing any variations from the normal in the behavior of the cell.

A. Gruber (1886) was perhaps the first to cut an amœba into two pieces for the purpose of observing the effect of the removal of the nucleus upon locomotion. He says (free translation), "The part with the nucleus continues to project and withdraw its pseudopods, as before but the part without a nucleus withdraws its pseudopods although there is a weak streaming at first. Two days after the bisection the (enucleated) amœba died." He concluded that the experiment showed that in amœbæ the capacity for movement is affected, which he had found was not true in the case of the Infusoria. Hofer (1890) found in a large number of experiments that the removal of the nucleus exerted a direct influence upon the movement of the protoplasm, and he therefore concluded that the nucleus is a regulatory centrum for the locomotion of the amœba. He also found that enucleated amœbæ ceased to form the secretion wherewith amœbæ attach themselves to the substratum. His descriptions and figures show that at no time did the fragments free from nuclear influence approach normal locomotion. Willis (1916) observed a number of enucleated amœbæ for seventy-two hours. He observed that during this time their movements were jerky, irregular and very much slower than those of nucleated parts. They were able to attach themselves to the substratum only weakly and for short intervals of time. Lynch (1919) found that the enucleated part, seven minutes after amputation, ceased its progressive movements and retracted into a corrugated sphere. After one or two days some of the nucleated fragments commenced to move characteristically, but were not able to attach themselves to the slide. Against all this evidence, we have the work of Stolć (1910) who reaches the rather amazing verdict that enucleated amœbæ show the same characteristic movement as nucleated ones, including the feature of attaching themselves to the substratum. Furthermore, he showed to his own satisfaction that enucleated amœbæ were as irritable as nucleated ones, and exhibited the same stimulated condition.

It is evident that it is impossible to gain from the literature any adequate conception of the bearing of the nucleus upon the locomotion of amœbæ. It was for this reason that the writer undertook the experiments reported in this paper. The amœba employed throughout was *Amœba dubia* which is characterized by an ovoid nucleus, lack of ectoplasmic ridges, and the possession of many pseudopods, with no axial pseudopod. Thanks are due to Dr. J. A. Dawson for giving assistance in way of amœbæ and technique in starting the cultures. After the cultures were once well started, little difficulty was experienced in carrying them on.

From their figures, it appears that Hofer used the type of amœba with the ovoid nucleus, possibly *A. dubia*, while Stolć used an amœba with a biconcave nucleus, probably *A. proteus* or *A. discoides* (See Schaeffer, 1916). The others employed "*A. proteus*," but from their figures it is impossible to determine which species of the *proteus* types.

The instrument employed in my experiments in removing the nucleus was a fine, hard glass point drawn out in a micro-burner, by means of which the portion of the amœba containing the nucleus was severed from the remaining portion. In all cases the enucleated portion was slightly larger than the nucleated. Each portion of the amœba was then placed in a watch glass in a large drop of fluid from a culture in which amœbæ were actively growing, and kept in a moist chamber for observation. The water was changed twice daily so as to be kept as fresh as possible.

Records were kept of sixty-four enucleation experiments, although many more were performed with similar results. The length of life of the enucleated fragments was somewhat variable, as follows:

Two amœbæ lived only one day; eleven, two days; eighteen, three days; sixteen, four days; eleven, five days; four, six days; one, seven days; and one lived eight days. Thus the greater number lived between three and four days. This may be compared with Hofer's enucleated amœbæ which lived from eight to twelve days (one more than fifteen days) and those of Lynch which lived for from six to eight days. Stolć claims to have been successful in keeping amœbæ artificially enucleated alive for as much as twenty-five days. Strangely enough, however, two enucleated fragments which arose from two different amœbæ that had divided spontaneously lived only ten and four days respectively (Stolć, 1910). It is possible that some of the variations in length of life are due to the species of amœbæ employed by the various workers.

In order to ascertain the effect of the removal of the nucleus upon the locomotion of the amœbæ, sixty-four experiments were performed, and carefully recorded. Each amœba was under observation for a few hours after cutting, and was studied at frequent intervals afterwards so long as it showed any activity. The following experiment is typical of the greater number of the experiments and will be given somewhat in detail.

An amœba moving actively in a healthy culture (Fig. 1) in a Syracuse watch glass was bisected at 3:44 so as to stimulate it as little as possible. The protoplasmic streaming was not discontinued in either piece (Figs. 2 and 3). The fragments moved away, however, in opposite directions from the plane of cleavage, as invariably happens when a clean cut is made so as not to agitate the amœba too much. At 3:45 both portions were still moving, attached to the substratum, as determined by directing a fine stream of water from a pipette upon them. At 3:49 both were still progressing normally. But from 3:49 to 3:54 the activity of the enucleated part slowly diminished (Fig. 4), it lost its hold on the substratum, and the extended pseudopods appeared literally to shrink into short, stubby, wart-like processes (Fig. 5). Under the higher power of the microscope it was apparent that the surface of the fragment was wrinkled and that the clear zone of ectoplasm around the periphery had disappeared, the granular protoplasm extending throughout. Meanwhile the nucleated portion continued to move quite naturally. Later it was placed in a moist chamber where it ingested food and divided two days later. From 3:54 to 5:23 the enucleated fragment remained in the irregularly spherical shape, with the surface considerably wrinkled (Fig. 6). The shape changed somewhat from time to time, but almost imperceptibly. At 5:23 it sent out a long pseudopod (Fig. 7) and commenced a slow streaming, but because it was not attached to the substratum there was no progressive movement. At six o'clock it was attached and streaming actively in an irregular limax fashion (Fig. 8). During the next two days it was attached and streaming actively in a *limax* fashion when observed (Fig. 9). On the third day the streaming was slower, change of shape was not so rapid, the attachment to the substratum was loosened, and the periphery of the amœba was a wide zone of clear ectoplasm (Fig. 10). On the fourth day perceptible streaming and change of shape had ceased, and the amœba was called dead.

From the series of experiments, certain definite facts were learned. First, locomotion is not at once affected by the removal of the nucleus if the amœba is not agitated in the process of cutting. Second, from five to ten minutes after enucleation the amœba commences to stream more slowly. This is accompanied by a gradual release of its hold on the substratum. Third, the surface of the enucleated portion becomes wrinkled, as if the pressure from within were reduced. Lynch too mentions the fact that his amœbæ assumed the shape of a corrugated sphere about seven minutes after the removal of the nucleus· Hofer's figures show that he too obtained it. It appears as if the removal of the nucleus causes the cell to lose turgidity. Entire amœbæ placed in 20 per cent. sugar solution become wrinkled in a similar manner after about fifteen minutes. Third, in about two and one half to three hours after enucleation the streaming is resumed, the amœba attaches itself to the substratum, and moves in a manner approximating normal streaming. More often, however, it assumes a *limax* shape, and moves after the fashion of a monopodal amœba, sometimes forming lateral pseudopods, but only temporarily. Fig. 16 shows an enucleated amœba moving in an extremely normal fashion two days after merotomy. Concerning the causes why the amœba should again become active after an interval of comparative inactivity, one can only speculate. There must be a certain amount of pressure within an amœba before it can project a pseudopod. Mast says that it is the contraction of the plasmagel and the hypertonicity of the plasmasol which provides the mechanics of locomotion. The pressure within is reduced when the nucleus is removed, but is increased to some extent after an interval of two to three hours. Certainly then the nucleus is not the centrum for controlling the locomotion of the amœba. Rather, due to some unknown physical cause, its removal seems to affect the imbibition of water into the protoplasm. Later the property of imbibition is reacquired. If the contractile vacuole is in the enucleated fragment at the time of enucleation, it disappears completely when the fragment becomes wrinkled (Figs. 5 and 6) and does not again appear until streaming is resumed (Fig. 7). Fourth, enucleated fragments, often show a tendency to move in a limax or monopodal fashion. Fifth, the removal of the nucleus does not affect noticeably the ability of the amœba to adhere to the substratum and thus approximate normal locomotion. Sixth, the solation-gelation (Mast 1923) process continues in enucleated amœbæ.

It should be noted, however, that in many cases where to casual observation it appears that an enucleated fragment is moving normally, a closer scrutiny and comparison with a normal amœba shows that the movement is not quite natural. It is difficult to get any quantitative data regarding the degree of normalcy of amœboid organisms. The tendency to move in a *limax* fashion, which *A. dubia* rarely does normally, is the most perceptible deviation from the normal.

There are other irregularities which one finds, such as the tendency of the enucleated *limax* form to flow in one direction for a while, and then very suddenly contract at the anterior end and flow in the opposite direction. Occasionally an amœba will flow in opposite directions from the middle for a short time, in which case the neck of protoplasm connecting the two streaming units becomes very thin. These phenomena seldom occur in normal nucleated specimens.

Stolć has made the assertion, and laid much stress upon it, that enucleated amœbæ show the same irritability as nucleated ones. That is to say, they show what he has designated as the equilibrium condition when streaming unmolested (Fig. 1.), and the stimulated condition when stimulated mechanically (Fig. 11). In experiments like the one described above, the amœbæ were not disturbed for a while after cutting. A number of experiments were performed in which the amœbæ were disturbed just after cutting and at other times. The following is a typical experiment.

At 3:12 an amœba was cut into two approximately equal pieces. They were drawn up into a pipette and transferred to another watch glass. Both went into the typical stellate stimulated condition (Figs. 11 and 12). At 3:20 the nucleated portion (*A*) was still suspended in the water in the typical stimulated condition. The enucleated portion (*B*) had lost all but two of its processes, and the entire surface of the amœba was rough and wrinkled (Fig. 13). At 3:26 (*A*) was still stimulated, but (*B*) had withdrawn into a wrinkled sphere (Fig. 14). At 3:30 (*A*) was about the same, but (*B*) had sent out a pseudopod with a rough surface. At 3:40 (*A*) was attached and flowing normally. (*B*) was still in a wrinkled condition but with a long wrinkled pseudopod (Fig. 15). At 3:50 both were again drawn into a pipette and shot out again in the stream of water. Both went into the stimulated (stellate) condition, but in just an instant (*B*) became again a roughened sphere. (*A*) remained in stimulated condition for the next ten minutes. At 4:45 both were again drawn into the pipette and shot out in a stream of water. (*A*) showed the typical stimulated condition, but (*B*) went at once into a wrinkled sphere. At no time during the three days of life of the enucleated portion (*B*) could it be made to respond to the stimulus in the normal manner, as did (*A*), which responded in the same manner as the normal entire amœba.

Thus it is apparent that at first both portions show the typical stimulated condition, but the enucleated portion loses the normal response withing a short time. It can respond only by forming a wrinkled sphere, as if it were unable to exert sufficient pressure from within to support the pseudopods. If one watches the "arms" of the stellate enucleated fragment after the initial stimulation following soon after the operation of cutting the amœba into two pieces, the arms appear to melt away, the surface being thrown into folds. The writer can offer no better explanation than that the degree of turgidity becomes insufficient to sustain the pseudopods. Thus we see that the reactions of amœbæ to mechanical stimuli are very much affected, the verdict of Stolć to the contrary. The enucleated fragment has not lost its irritability, but it no longer gives the normal response.

The nutritional processes of amœbæ, especially the ingestion and digestion of food have been further points of controversy.

According to Hofer, Lynch and others, enucleated fragments of amœbæ do not ingest food. Stolć declares that amœbæ ingest food in a perfectly normal manner for many days after enucleation. Schaffer (1920) has pointed out that feeding calls for concerted streaming, and whether enucleated amœbæ ingest food or not is certainly of great importance in evaluating the importance of the nucleus in cell regulation of amœbæ.

Figure 17 shows an *A. dubia* in the normal act of feeding with the interesting observation of one smaller food cup superimposed upon one arm of a large food cup. In culture fluids swarming with *Chilomonas* and *Euglenæ*, the writer has never observed an enucleated amœba ingest a food substance by means of a food cup. It has been observed that such a fragment may flow over a *Chilomonas*, when it appears as if a small cup were formed in the under side of the pseudopod, but on no occasion was the act of enclosing the flagellate and engulfing it into the protoplasm completed.

Schaeffer (1922) has mentioned that by stimulating an amœba with a fine glass rod by waving it in the water some distance from the amœba, the amœba may form a food cup (Fig. 27). While this reaction is not difficult to produce in the case of an entire, healthy amœba, in the case of an enucleated amœba there is either no reaction to a vibrating needle, or only an abortive pseudopod is extended (Fig. 28).

Although enucleated amœbæ which had ingested very large *Euglenæ* could often be found, one was never observed in the act of feeding. Once the writer put a large enucleated fragment of an amœba in a drop of water containing many *Euglenæ* and sat down at the microscope to watch it until the process of feeding should be observed. It was noted that *Euglenæ* which had no flagella often approached the amœba by the typical "euglenoid movement," but the amœba did not react positively toward it. After about an hour it was observed that an *Euglena* was battering the amœba in its attempts to go forward (Fig. 18). Instead of showing an "avoiding reaction," it would simply recoil, and make a new attempt to go forward in the same direction. Soon it had literally battered an entrance into the amœba (Figs. 18, 19, 20, 21). Finally the channel was deep enough to enclose the *Euglena*. The protoplasm of the amœba closed over the entrance, the *Euglena* struggled a bit, then doubled up, and a food vacuole was formed around it (Fig. 22). Such ingested *Euglenæ* are often digested, although sometimes they are egested very soon after entering the amœba. The process of digestion can be followed. First the *Euglena* gradually loses its green color, becoming brown in an hour or so; then it is reduced in size, and finally after many hours only some brown undigested remains are left in the food vacuole. The vacuole shows the acid reaction up to the end of the process, as proved by its intense redness to a very weak neutral red solution in the water.

Thus, while normal feeding reactions have never been observed in enucleated amœbæ, nevertheless, food may be ingested by an amœba, the organism ingested entering the amœba by its own efforts, and not by any positive activity on the part of the amœba. This method of feeding certainly does not call for concerted streaming. The inability of the amœba to form a food cup seems to be due to the lack of pressure from within sufficient to sustain the pseudopods engaged in the circumvalation of the food particle. Food once ingested can be digested by the amœba after the fashion of normal amœbæ.

In five instances not included in the sixty-four experiments, the enucleated fragment of the amœba had divided spontaneously in from twelve to forty-eight hours after merotomy. The writer chanced to see one such fragment stretched out as in Fig. 23. The streaming was essentially like that of two *limax* amœbæ jointed at their posterior ends. The neck joining the two became narrower and narrower, until the edges rounded off (Fig. 24), leaving two fragments moving in a *limax* fashion (Figs. 25 and 26).

While this is not, of course, normal cell division, it is interesting to note that a cell without a nucleus may divide. The division appeared to be due to the pull exerted in this region by the two halves tending to move in opposite directions, and an accompanying increase of surface tension at the point of division.

Conclusions.

Enucleated amœbæ of the species *Amœba dubia* show the following properties:

1. Within a few minutes after enucleation streaming ceases, and the amœba becomes a wrinkled sphere.

2. In a few hours the streaming is resumed.

3. This streaming is usually of the *limax* type, though it may approximate the normal.

4. Enucleated amœbæ may attach themselves to the substratum.

5. Enucleated amœbæ are irritable, but do not show the same response to stimuli which nucleated amœbæ show.

6. Food organisms which enters the body of an enucleated amœba are killed and digested in an apparently normal manner.

7. Enucleated amœbæ may become divided by antagonistic streaming currents within.

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DESCRIPTION OF PLATE.

(Free-hand drawings of actual specimens, except where stated otherwise.)

1. *Amœba dubia* in normal locomotion.

2. The fragment containing the nucleus, immediately after merotomy.

3. The fragment without a nucleus, containing the contractile vacuole.

4. Enucleated fragment in which streaming is arrested. The hold on the substratum is lost, and withdrawal of pseudopods and wrinkling of the surface has commenced.

5 and 6. The same fragment in the wrinkled condition. The contractile vacuole is not visible in this condition.

7. Streaming and pseudopod formation resumed, but not yet attached to substratum. Contractile vacuole again appears.

8. Streaming of enucleated fragment with pseudopod formation.

9. Limax streaming with small lateral branching, typical of enucleated fragments.

10. Enucleated fragment, practically dead.

11 and 12. Both fragments in stimulated condition.

13. Transitional stage to 14.

14. Wrinkled sphere appearing soon after typical stimulated condition in enucleated fragments.

15. Projection of pseudopod.

16. An enucleated amœba, moving in an almost normal manner, which has ingested and partially digested a number of Euglenæ.

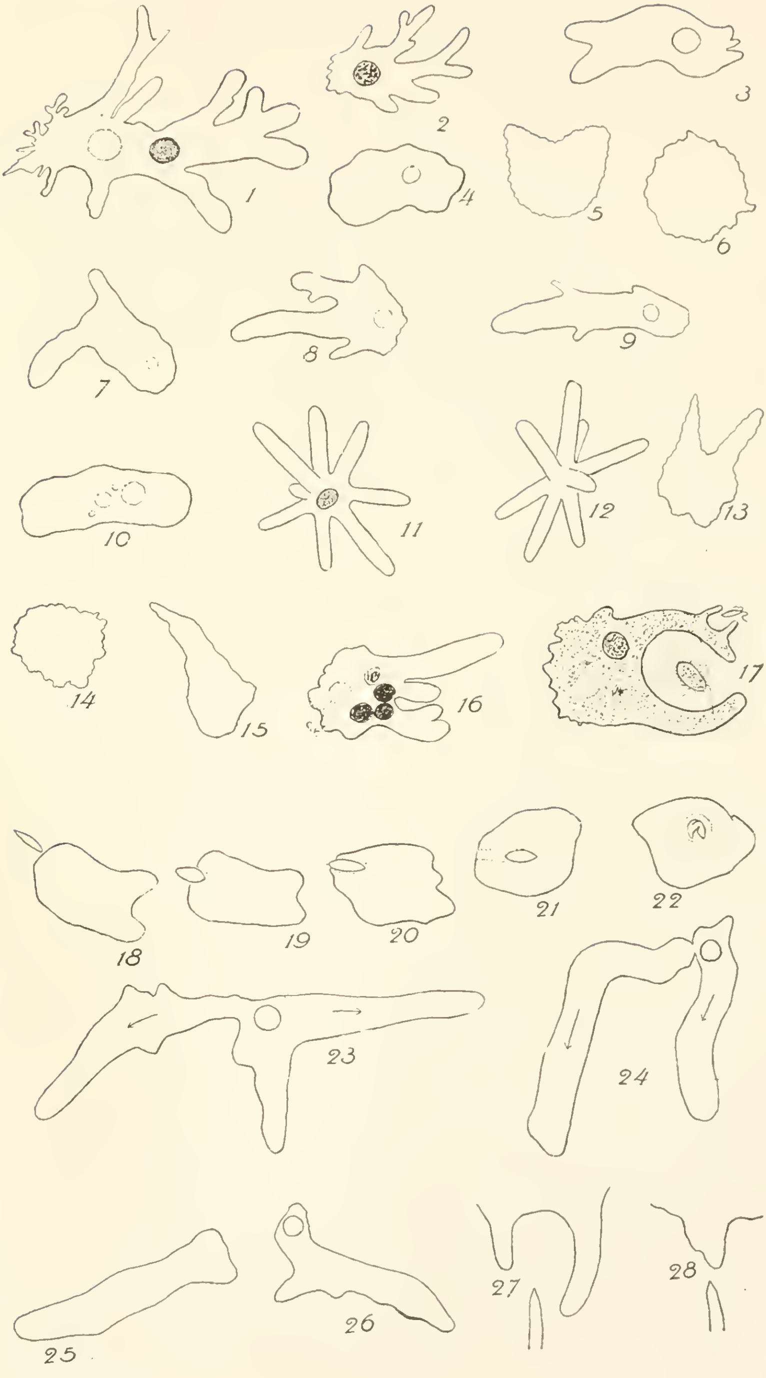
17. Food cup superimposed on a food cup of normal amœba in act of feeding.

18 to 22. An *Euglena* entering an enucleated amœba with final food vacuole formation.

23 to 26. An enucleated amœba dividing autonomously (camera lucida drawings).

27. Food cup induced in an entire amœba by agitating tip of glass rod in water.

28. Abortive reaction of enucleated amœba to the same stimulus.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ELERY R. BECKER.

PALM AND SOLE STUDIES.

IX. The Morphology of the Hypothenar of the Hand; a Study in the Variation and Degeneration of a Typical Pattern.

HARRIS H. WILDER.

Basing our conclusions upon the results of morphological observation, mainly those of Miss Whipple, 1904, it is safe to assume that a friction-skin pattern, whether a "finger-print," *i.e.*, one taken from the apical mound of a finger, or one located upon the broader surface of either palm or sole, has its origin in a raised conical mound, surrounded by an encircling duplicature of skin (Fig. 1). This assemblage of parts becomes covered by series of epidermic ridges, themselves composed of rows of single units, probably the morphological equivalents of scales, which in their configuration are influenced by the underlying details. The encircling folds develop as similar ridges which follow the edges, and the two, three, or four points where different folds meet each other form triangular fields, or often triradiate lines diverging from centers, the entire system of which precisely

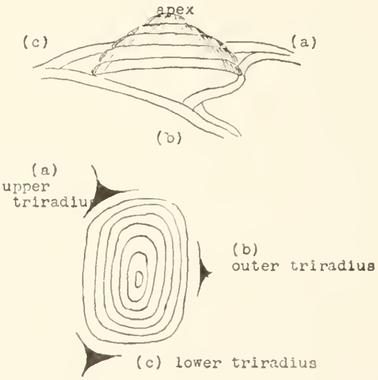

Fig. 1. Diagrams of a pad, and its enclosing folds showing apex, concentric circles and triradii.
Above—Profile view.
Below—Ground plan of same.

encloses and defines the pattern areas, upon which the definite patterns are developed. These latter, in typical cases, form in a definite relation to the original conical mound, and develop in concentric circles about the apex of the cone, with the center, or "core," of the pattern exactly coincident with this apex.

When, in the Primates, the height of these structures, that is, folds and mounds, becomes reduced to a flat surface, the relief becomes transformed into a picture, and the mound, with its enwrapping folds, becomes distinctly traceable in the ridges, the pattern area covered with concentric circles, and the outer contour interrupted by triradii. Two radiants of each triradius, extend in nearly opposite directions and embrace, or frame in, a part of the perimeter of the pattern, while a third, the divergent radiant, passes off centrifugally from the pattern.

Those patterns which are located upon the surface of the palms or soles, have each three of these embracing triradii, save the Third of the Interdigital series of the hand, which has four, but the apical patterns, doubtless because of the rounded terminations of the ends of the digits, are furnished with two only, the lateral ones, while the ridges which terminate the digits continue to follow around the contour of the digit, and dispense with a third triradius.

The arrangement of each pattern and its surroundings, including the number of triradii originally embracing each pattern, and their relative position, may be seen by a diagram, published several times, to which the reader is referred.[1] Aside from this the conditions seen in generalized quadrupedal mammals, as shown in the 1904 paper of Miss Whipple,[2] and from which this key diagram was deduced, is of fundamental interest. See especially the paws of *Microtus*, pp. 270 and 272, that of *Crocidura* on p. 280, and the generalized diagram of this author on p. 275, in which each radiant of each triradius is named.

To select a good typical pattern, the transformations of which could be conveniently studied, we may propose the *hypothenar of the hand* (Fig. 2) for the following distinct advantages:

1. It is a pattern with three typical triradii, and consequently three divergents. It is thus better than any finger-print.

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[1] "Palm and Sole Studies," BIOL. BULL., Feb., 1916, Figs. 3–5, p. 142–144.

[2] *Zeitschr. f. Morph. u. Anthrop.*, Bd. VII., 1904.

2. It is a large pattern, not placed very near any other, and hence so far beyond the influence of others that we are seldom at a loss to decide whether a certain feature belongs to it or to an adjacent pattern.

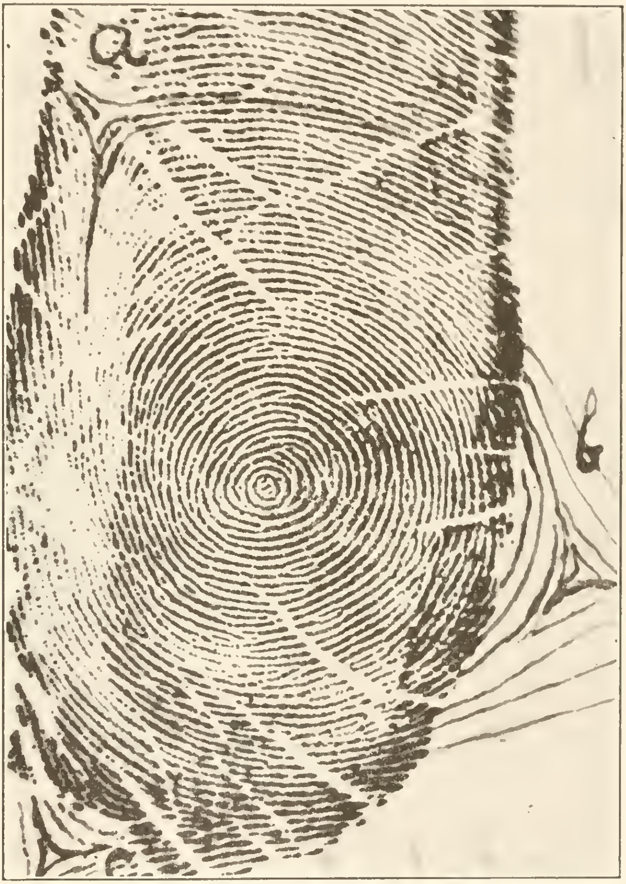

FIG. 2. Actual print of a primitive hypothenar. Triradii *a* and *c* are shown in the print; triradius *b* is indicated by the curve of the friction-ridges and is completed by pencil lines.

3. The region where it occurs is covered by large, heavy ridges, the prints of which are among the most distinct upon the entire palm.

4. This pattern presents an unusual number of variations, and is of fairly common occurrence, rendering it easily possible to collect a large amount of material.

Referring again to any of these diagrams, it will be seen that the pattern in its typical form is centered upon about the middle of the hypothenar region of the hand, and that its three triradii consist typically of (*a*), an *inner upper*, (*c*), an *inner lower*, and (*b*), an *outer*, the first situated near the hollow in the center of the palm, the second down upon the wrist near its middle line, and the third far around upon the outside edge of the hand. This last is, indeed, frequently placed so far around the hand as to be *extralimital*, that is, placed beyond the limits of the true friction-skin, and indicated only by the convergence of the friction ridges along the transition zone between friction-skin and the normal skin of the back of the hand.

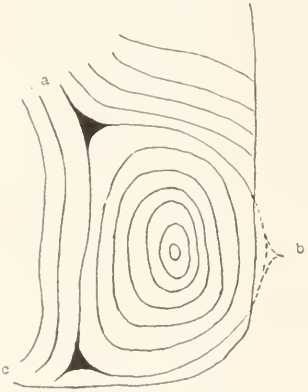

FIG. 3

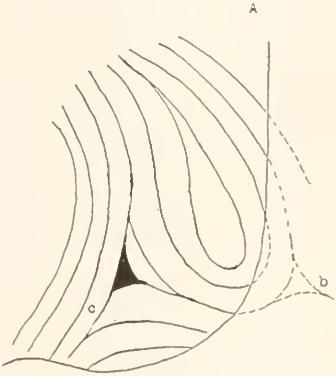

FIG. 4

FIG. 3. A typical hypothenar, based on Fig. 2, an actual print.

FIG. 4. A hypothenar pattern, in which triradius *a* is wanting, and the ridges escape in that direction. This is type *A*.

It is now possible to imagine any one of these three triradii degenerating, and this degeneracy going so far as to allow the escape of the ridges that are normally held in place by its two embracing radiants. Such a loss would convert the concentric circles, a WHORL pattern, into a typical LOOP pattern, open to the point from which the given triradius has disappeared; and as these three triradii are designated as *a*, *b*, and *c*, (Fig. 3) the three resulting loops may receive their names from the triradius that has broken down in each case, and we have types *A*, *B*, and *C*.

In type *A* (Fig. 4) triradius *a* is wanting, while *b* and *c* persist, thus forming a loop which opens obliquely up across the palm, following the "Line of Life" and pointing up towards the base of the index finger. Type *B* (Fig. 5) opens outwards towards the outer margin, where triradius *b* is wanting, and thus allows the figure to open widely on this side. Triradii *a* and *c* are persistent. In type *C* (Fig. 6) the persistent triradii are *a* and *b*, while the failing one is *c*, the lower medial one. This results in the formation of a loop that runs obliquely downwards and medially, pointing towards the middle of the wrist.

Aside from these three simple types, which result from the loss of a single triradius, there may be found those that result from the loss of two out of the three triradii. In these the final result is not a loop, but a lenticular or crescentic figure, tapering at the two ends of a prolonged axis. In type *AC* (Fig. 7) the axis of the figure runs in a curve from above, where it tapers to the hollow of the hand, down to the middle of the wrist, where it tapers again; in type *AB* (Fig. 8) a similar figure is placed obliquely across the hypothenar eminence from upper medial to lower lateral, retaining triradius *c*.

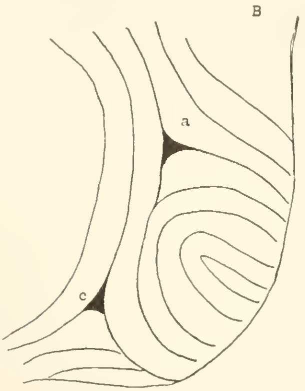

FIG. 5

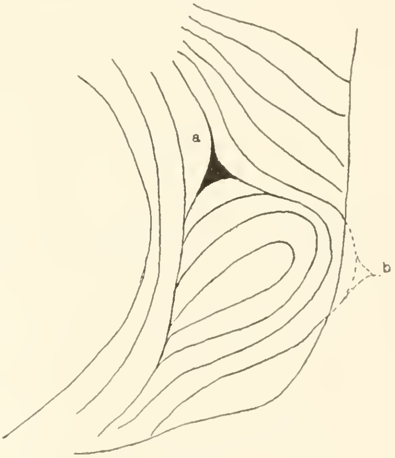

FIG. 6

FIG. 5. A hypothenar pattern, in which triradius *b* is wanting, and the ridges escape in that direction, forming type *B*.

FIG. 6. A hypothenar pattern, in which triradius *c* is wanting, and the ridges escape in that direction, forming type *C*.

At the present moment I am not quite sure whether type *BC*

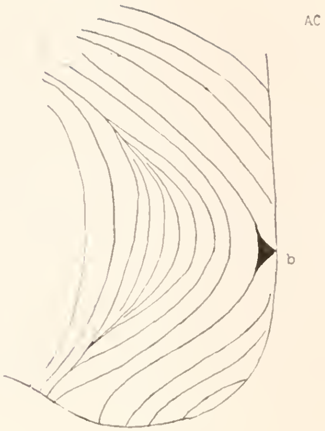

FIG. 7.

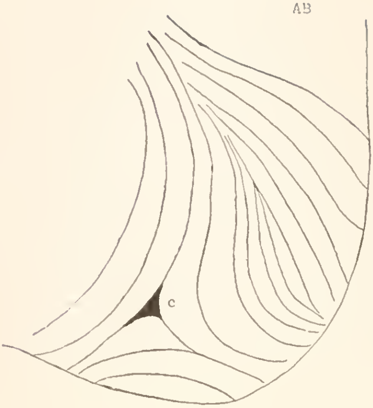

FIG. 8.

FIG. 7. A hypothenar pattern, in which both triradius *a* and triradius *c* are wanting, and the ridges escape in these two directions. This is type *AC*.

FIG. 8. A hypothenar pattern, in which both triradius *a* and triradius *b* are wanting, making type *AB*.

occurs or not, but there seems no reason why it should not. This type (Fig. 9) should retain triradius *a*, the radiants of which should embrace a lenticular figure, shaped like the two others, but with its longitudinal axis running obliquely across the lower outer corner of the palm and tapering down to the points where one normally expects the two triradii, *b* and *c*.

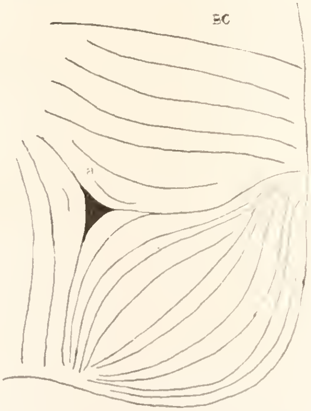

FIG. 9. A hypothenar pattern, in which both triradius *b* and triradius *c* are wanting, forming type *BC*.

Thus, starting with the primitive whorl, and adding the three types that arise from the loss of each of the triradii, one at a time, and also the three arising from the simultaneous loss of any two, we have *seven primary types*, in which the variations are due to differences in the originally embracing folds, with their triradii,

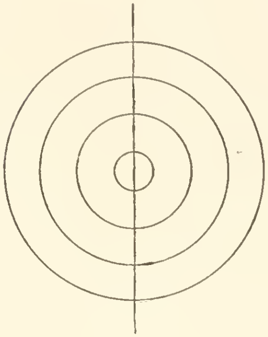

FIG. 10

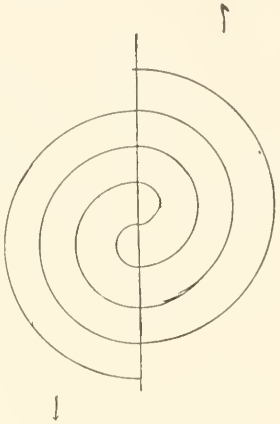

FIG. 11

FIG. 10. A typical whorl pattern, composed of concentric circles. By cutting this through the center, as along the line, and then sliding the two halves as in Fig. 11, an S-shaped spiral is produced.

FIG. 11. An S-shaped spiral the result of sliding the two halves of Fig. 10, as explained in the text. This form is frequent in all kinds of patterns.

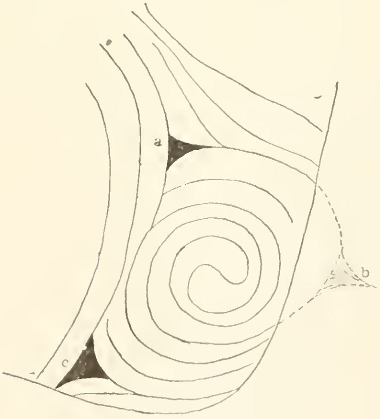

FIG. 12

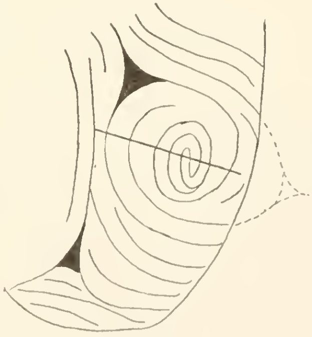

FIG. 13.

FIG. 12. An S-shaped pattern surrounded by its defining triradii.
FIG. 13. An S-shaped pattern, showing the axis along which the slipping may have taken place.

and have nothing to do primarily with the ridges of the pattern itself.

Turning now to the latter there seems to be one distinct line of modification affecting the center of the Whorl; the effect of a shoving of the two halves in opposite directions. This action is most readily seen, and its effect comprehended, by drawing out upon a card a series of concentric circles of the same width, cutting the figure in two through the center, and then slipping the two parts on each other to any distance desired.

Frequently this slipping past of the ridges upon the two sides affects only the central portion of the pattern area, giving a result quite like an ordinary Whorl, with an S in the center (Fig. 12). In such cases it remains only to be determined the axis of the slipping, that is, the line along which the diagram of Fig. 10 must be cut in order to slip in such a way as to produce the exact pattern we have before us (Figs. 13, 14, and 15).

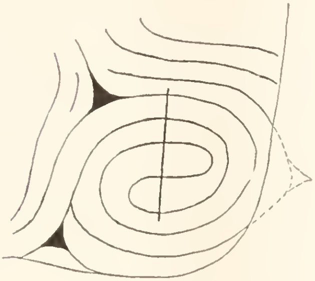

FIG. 14

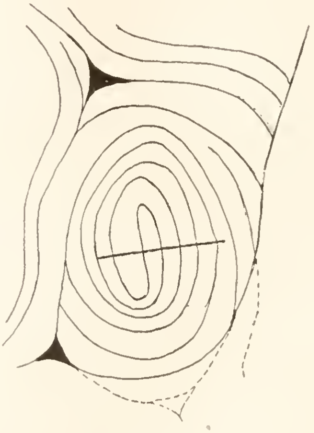

FIG. 15

FIG. 14. An S-shaped pattern, showing an axis of slipping unlike that of Fig. 13.
FIG. 15. An S-shaped pattern, showing an axis of slipping unlike those shown in Figs. 13 and 14.

In other cases, however, where the extension of the slipping spreads the two half-loops further and further apart, the pattern may become spread out so far that the S-shaped figure covers practically the entire hypothenar area, thus producing an actual double figure, or a figure with two distinct loops, and even a distinct separation between them. In these cases there may appear a new triradius between the two parts, a "degeneration triradius," resulting from this process, and having no counterpart in any element of the primary pattern or its surroundings (Fig. 16).

The two portions of this outspread pattern seem to develop without any correlation; the upper one may remain as a large loop (Fig. 18) or may disappear (Fig. 17, 19). Similarly the

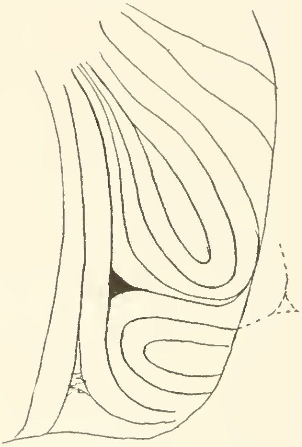

FIG. 16. An S-shaped pattern, which is so large that there has developed a "degeneration-triradius," which cuts the pattern in two and makes two loops of it. Each loop is apparently independent and is directed in different directions.

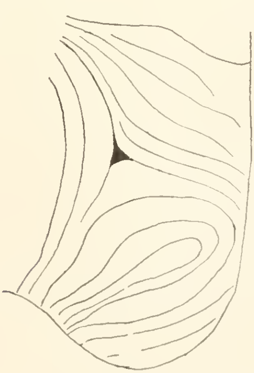

FIG. 17

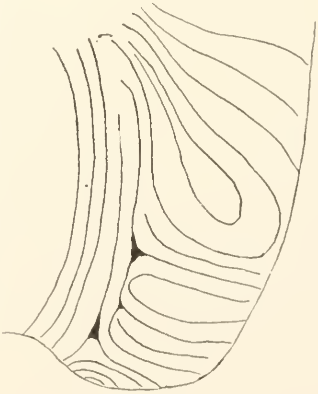

FIG. 18

FIG. 17. A double S-shaped pattern, in which the loops differ in direction from those shown in Fig. 16.

FIG. 18. A double S-shaped pattern with the two loops again unlike those of Figs. 16 and 17.

lower one may appear as a loop, either facing in (Fig. 17) or out (Figs. 18, and 19). Finally the two parts may both become entirely effaced (Fig. 20) the last sign of the former pattern being the degeneration triradius, with the separation of the area of otherwise parallel lines into the two parts of an original S-shaped pattern.

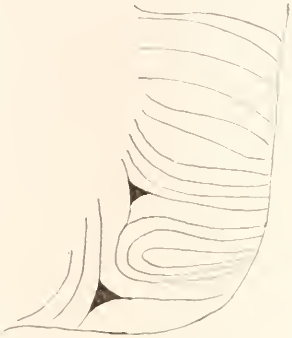

FIG. 19. A double S-shaped pattern, in which the upper loop has disappeared, and is represented by lines that show no trace of a curve.

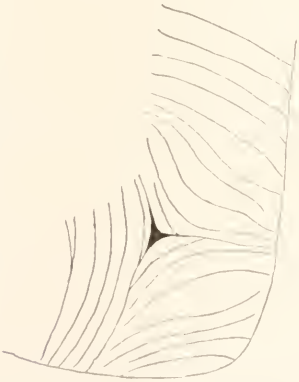

FIG. 20. This shows the ridges covering a hypothenar region, covered by parallel cross-lines, but showing a degeneration triradius, dividing the entire area into halves. It may be supposed that during the evolution of this form it may be evolved from an S-shaped spiral pattern, which has developed a degeneration triradius, and that each pattern of this has degenerated completely.

It is possible to bring into the category of spread-out S-patterns certain cases that consist simply of a rather small and narrow loop, placed very low down on the palm, adjacent to the carpal margin (Fig. 21). This loop suggests the lower half of the double

S pattern, with the loop facing as in Fig. 17, but eventually curving upwards instead of downwards. There may be, in these cases no trace of either a degeneration triradius or of the separation of the whole area into the two halves resulting from an elongated S, and it may be simpler to refer this form to a simple *A* loop of an unusual shape and position.

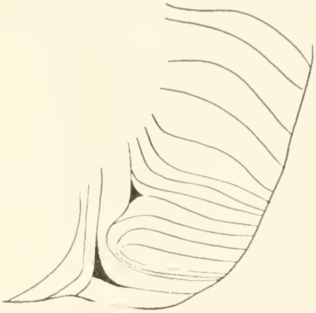

FIG. 21. In this area there is a degeneration triradius, and the lower pattern has retained its loop. This suggests an explanation for a common type, in which the hypothenar region is made of parallel lines, but has a small loop very low down.

The Migration of Triradii.—Thus far we have contented ourselves with the selection, and somewhat with the classification, of types of patterns, and have separated and grouped the following:

W—The Whorl Type (primitive).

A—The *A*-Type, a loop opening upward.

B—The *B*-Type, a loop opening laterally.

C—The *C*-Type, a loop opening downwards and inwards.

AB—The *AB* Type, a lenticular figure, extending between the positions of triradii *a* and *b*, but with the triradii themselves represented only by converging lines, and with the two embracing radiants wanting.

AC—The *AC* Type, same as before but with the missing triradii *a* and *c*, and represented only by converging lines in the two positions.

BC—The *BC* Type, same as the two foregoing, but with the missing triradii *b* and *c*.

S—The *S*-Type, a whorl with an S in the center. This S figure may be confined to merely a few of the central circles, or may be more extensive. It may be elongated to cover the entire area, and its axis may be placed at any angle.

D—The Two Loop Type, developed from an elongated S, the two parts of which are separate and distinct from each other, and separated by a "degeneration triradius." In subdivisions of this type the two original loops may develop independently of each other.

It has been suggested by the method of description of these types that triradii do degenerate, commonly forming converging lines without lateral radiants; it is also assumed that they migrate in position. In this latter case the movement may be either *centripetal*, the two lateral radiants approaching the center, or *centrifugal*, the lateral radiants allowing more and more complete rings to intervene between the center or core of the pattern and the center of the triradius. In this latter case the two lateral radiants naturally meet at a sharper and sharper angle until eventually they become lost in the set of converging ridges, and are not to be distinguished from the rest. All stages in these two processes are seen in individual cases, although, as no changes are to be found during the life of an individual the process can only be inferred. Thus to start with a case of centripetal movement we can see this only by finding a series of stages, each permanent for a given individual but representing steps in the process carried on by the race. Whether these steps may be found to be taken phylogenetically along a line of descent is not known as yet.

Thus far, in some 3400 human hands, there has been found an hypothenar pattern that does not belong easily to one of the types described here; I found in a Japanese male a hypothenar that cannot well be thus included, and I wish to present it here without further explanation (Fig. 22). It evidently corresponds closely to the types in which the entire pattern is divided by a "degeneration triradius" into two parts; but where in the most of these, each part may be found in the form of either a loop or a completely degenerated area in which it is covered with a series of parallel ridges without trace of a pattern, the upper half of this one, instead of being a loop as might be expected, is in itself a perfect Whorl, like the pattern we commenced with while below this there is a complete loop. I have looked at this anomalous pattern again and again, without coming any nearer to any explanation. I have therefore given it as it is, an actual pattern which cannot be explained, and the only one out of several thousands. The explanation may occur to some one who reads this paper, or it may well represent a new type, which will require some new modification of the primary pattern; yet it may hardly lead to the abandonment of the fundamental plan of a pattern, which certainly rests upon too much that is fundamental to suggest so radical a procedure.

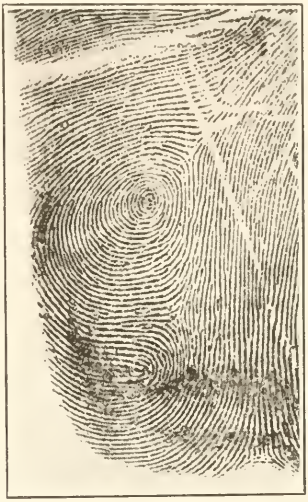

FIG. 22. A wholly excentric hypothenar region found in the left hand of a Japanese. The pattern is double, but instead of each consisting of a loop, degenerate or otherwise, one of them is a loop while the other is a whorl, the type from which we began in the explanation of all double types. It is possible, on the other hand, that we may have here another explanation in which we have to begin with two whorls, divided by a triradius, like the one given here.

THE AXIAL GRADIENTS IN HYDROZOA. VIII. RESPIRATORY DIFFERENCES ALONG THE AXIS IN TUBULARIA WITH SOME REMARKS ON REGENERATION RATE.

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AXIAL DIFFERENCES IN OXYGEN CONSUMPTION.

The experiments recorded in this section were performed at Swan's Island, Maine, in August, 1924. This island is one of a group off the coast of Maine, opposite Rockford, and is directly south of Mt. Desert Island. My visit to Swan's Island was occasioned through information received from the Anglers Company (now the Denoyer-Geppert Company) that *Tubularia* flourishes in that locality during the summer months, at which season, as is well known, it is in poor condition at Woods Hole. The company further kindly invited me to make use of their collecting station and equipment located on Swan's Island. I am greatly indebted to the company in all of these matters and particularly to Mr. Philip Turner, a member of the firm, for assistance in collecting *Tubularia*.

The collecting station at which these experiments were performed is situated in the town of Swan's Island, which town is located at the head of a large harbor and is provided with the usual wharves, pilings, etc. *Tubularia* was found in large quantities on the pilings of the steamer wharf and is apparently restricted to this one habitat. Members of the Anglers Company informed me that they had never seen it in any other situation nor on any other of the wharves or pilings. Large quantities of the hydroid were readily obtained at low tide from the piles supporting the wharf.

The *Tubularia* occurring at Swan's Island does not seem to be identical with the *T. crocea* of Woods Hole. The stems are much more elongated and branching less frequent. These characteristics may, however, result from the low temperature of the water, which did not, on the warmest summer days, exceed 15° C. The species is very favorable for experimental work, owing to the long unbranched stems and their freedom from other growths.

For the oxygen consumption large colonies were collected and from these a number of long straight clean unbranched stems were selected. They were carefully examined under the microscope to determine that they were free from diatoms or other organisms. In some cases a few such organisms were seen growing on the perisarc but it was found that these could be removed by gently brushing the stem with a camel's hair brush. The hydranth and upper two or three millimeters and the basal part of the stem were then cut off, leaving a clean stem twenty to thirty millimeters long. This was then cut into two pieces, the basal piece generally taken a little longer than the apical piece to compensate for its smaller diameter. Six to eight such pieces were used in each experiment and an attempt was made to select for each experiment stems of similar diameter. All stems used in any one experiment came from the same colony.

The method of determining the oxygen consumption was the same as previously employed in a similar study on *Grantia* (Hyman, '25). For details this paper should be consulted. Briefly small tubes detachable into two sections are employed. At the end of the experiment the pieces of stem are brought by gravity into one section, which is then removed; the other section is analyzed for oxygen content. In each experiment four tubes are employed: one containing the six to eight apical pieces of stem; one containing the basal pieces of the same stems; and two water blanks.

To compare the rate of oxygen consumption of two objects, it is necessary to know the quantity of protoplasm in each. Owing to the lack of a balance at Swan's Island, the weights of the pieces could not be determined. There would be some difficulty in determining the weight, owing to the presence of the lifeless perisarc around the stems; but some method could probably be devised to eliminate this difficulty. Under the circumstances, however, I was compelled to use the volume of the pieces as a standard of comparison. After each experiment the diameters of the stems were determined under the compound microscope with an ocular micrometer. However uniform a stem may appear to the eye naturally under the microscope considerable variations in diameter are perceived. Some fifteen to thirty measurements of the diameter of the cœnosarc were made at frequent intervals along each stem and these were averaged. The length of the piece was measured on a millimeter rule. From the average diameter and the length the volume of the piece was calculated assuming it to be a cylinder. No correction was made for the central cavity. The volumes of all of the apical pieces in any one experiment were added together and the volumes of the basal pieces similarly; and when the oxygen consumed is divided by these volumes, the quotients can be used to compare the rates of oxygen consumption of apical and basal pieces.

There is no doubt that considerable error is involved in such determinations of the volume of cœnosarc in the pieces. It does not seem to me, however, that the weight of the cœnosarc could be determined any more accurately. All determinations of the rate of respiratory metabolism are necessarily erroneous since there is no known way of discovering the actual quantity of respiring protoplasm in an organism. The consistent results which I have obtained in *Tubularia*, the definite relation noted between level and oxygen consumption, and diameter and oxygen consumption, indicate that the experiments are sufficiently accurate to render the conclusions acceptable.

Nine experiments were performed. The results are presented in Table I. The first column of figures in this table gives the oxygen content in cubic centimeters of the water in the tube at the beginning of each experiment; the second column the oxygen content in the tube at the end; and in the third column is given the difference between the first and second columns, or the oxygen consumed by the pieces. The data are presented in this way for the sake of simplicity; they are not actually obtained in this form as the original oxygen content of the tubes containing the pieces has to be calculated from the blanks. The differences in oxygen content of the two tubes in each experiment at the start are due simply to differences in the volumes of the tubes; for the same water is used in both tubes in each experiment. The fourth column of figures gives the total volume of the pieces in cubic millimeters, the fifth column the average diameter of the pieces, and the sixth column the oxygen consumed per cubic millimeter in the time occupied by the experiment. Each experiment lasted four hours. In the first experiment six pieces of stem were used; in the other experiments eight pieces. The temperature was 20° C. in experiments 1 to 3 and 16° C. in experiments 4 to 9. The volumes of oxygen as presented in the table have been corrected for these temperatures. The nine experiments are arranged in the table in the order of the diameters of the stems used, largest first, smallest last. As already stated,

TABLE I.

SHOWING THE CUBIC CENTIMETERS OF OXYGEN CONSUMED PER CUBIC MILLIMETER OF VOLUME IN FOUR HOURS BY APICAL AND BASAL PIECES OF THE STEM OF *Tubularia*.

Duration of all experiments, four hours.

No. of Exp.	Level of Pieces	O_2 Content at Start, cc.	O_2 Content at End, cc.	O_2 Consumed, cc.	Vol. of Cœnosarc, cu. mm.	Aver. Diam., mm.	O_2 Consumed per cu. mm. in Four Hrs.
4	Apical	.070	.061	.009	32.97	.63	.00027
	Basal	.060	.054	.006	30.84	.59	.00019
5	Apical	.068	.060	.008	24.71	.55	.00032
	Basal	.061	.055	.006	21.90	.53	.00027
3	Apical	.065	.055	.010	20.51	.58	.00048
	Basal	.060	.052	.008	18.68	.46	.00042
6	Apical	.065	.055	.010	20.34	.53	.00049
	Basal	.065	.057	.008	19.61	.51	.00040
7	Apical	.077	.067	.010	18.39	.50	.00054
	Basal	.067	.060	.007	19.25	.48	.00036
9	Apical	.072	.057	.015	20.25	.49	.00074
	Basal	.068	.057	.011	21.76	.51	.00050
8	Apical	.076	.061	.015	17.02	.46	.00088
	Basal	.071	.061	.010	15.08	.44	.00066
1	Apical	.068	.058	.010	11.48	.43	.00087
	Basal	.058	.052	.006	11.10	.41	.00053
2	Apical	.067	.056	.011	13.52	.38	.00081
	Basal	.063	.055	.008	14.59	.35	.00054

the six or eight stems in each experiment were selected so as to be of similar diameters.

The following generalizations may be drawn from the data given in Table I.:

1. In all cases the rate of oxygen consumption per unit volume is higher in the apical than in the basal pieces. This result is in harmony with differences in rate of regeneration and in electrical potential which exist along the stem of *Tubularia* (cf. Hyman, '20).

2. There is an inverse relation between the diameter of the stems used and the rate of oxygen consumption, the rate being higher the smaller the diameter of the stems. This agrees with the general law that in animals respiratory rate is inversely proportional to size.

3. In general it appears that the respiratory differences between apical and basal pieces are greater the smaller the diameter of the stem. This indicates that the respiratory gradient is steeper the more slender the stem.

These conclusions are considered further in the discussion.

Experiments on Regeneration.

Data on certain questions regarding regeneration which were obtained on *Tubularia marina* at Pacific Grove, California, in 1922, on *Tubularia crocea* at Woods Hole, Massachusetts, in 1924, and on *Tubularia* sp. at Swan's Island, Maine, in 1924 are herein presented. Owing to the large amount of work that has already been done on the regeneration of *Tubularia*, it does not seem necessary to present these data in detail. For the most part general statements will be made.

The expression "rate of regeneration" is defined and used to mean the time which elapses between the act of cutting and the attainment of a condition of equilibrium. Since in pieces of *Tubularia* of the size employed complete regeneration of a hydranth at the apical end of the piece always occurs, a condition of equilibrium is here synonymous with the completion of a hydranth. Practically, however, it is difficult or impossible to determine the exact time at which the regenerated hydranth is complete. As is well known, the new hydranth forms within the perisarc and the time of completion of its formation in situ would furnish the most accurate measure of rate of regeneration if such time could be determined. For practical purposes one generally notes the time at which the finished hydranth emerges from the top of the old perisarc. There is no doubt some error in such a procedure for in some cases at least such emergence may be greatly delayed by irregularities in the perisarc through which the hydranth must force its way. However, no better criterion of the time of completion of regeneration has been suggested by anyone and the time occupied by the regeneration process will here be used to signify the time between cutting and the emergence of the completed hydranth.

In all regeneration experiments straight healthy unbranched stems were employed. These were invariably cut as follows. The hydranth and upper millimeter or two and the basal portion of the stem were removed and discarded. The remaining stem was then cut as desired.

The regeneration of only the oral hydranth—*i.e.*, the hydranth which forms at the apical end of each piece—was studied. Unless specifically stated otherwise, all statements refer to this hydranth only. No study was made of the regeneration of aboral hydranths.

1. *Rate of Regeneration of Halves of the Stem.*—In such experiments the stem is divided into halves and the time between cutting and emergence of oral hydranths noted for apical and basal halves. As above stated the observations refer only to regenerated oral hydranths. In a previous paper (Hyman, '20) I presented a considerable mass of data on this matter using *Tubularia crocea.* These experiments showed that the time between cutting and emergence of oral hydranths is markedly shorter in apical than in basal halves. Since writing that paper the same result has been obtained with *T. marina* and with the Maine species. The result on *T. crocea* was also again verified. In Table II, I present a typical experiment on *T. marina.*

2. *Rate of Regeneration of Thirds of the Stem.*—A few experiments on this point were performed on *T. marina* and on the Maine species. The result was found to be different in the two species. In *T. marina,* the apical third regenerates first, the

middle third next, and the basal third last, the differences between the three pieces being well-marked. The basal third in *T. marina* is generally much behind the other pieces. A typical experiment on *T. marina* is given in Table II. On the contrary in the Maine species, the time between cutting and emergence of hydranths is about the same for the middle and basal thirds, the apical third being in advance as usual. I have, however, but five sets of pieces in which the time of emergence of the oral hydranths was exactly determined in all three pieces. These times are as follows, apical third first, middle third next, basal last: 39, 45, 46 hours; 41, 52, 52 hours; 42, 53, 53 hours; 43, 50, 53 hours; and 41, 46, 46 hours. These pieces were 8 to 10 mm. long.

Table II.

Regeneration Rate of Halves and Thirds of the Stem of *Tubularia marina*.

The first column gives the hours elapsed between cutting and emergence of the oral hydranths; and the other columns the number of hydranths emerged at the hours indicated. Length of halves, 3–7 mm.; length of thirds, 5–8 mm.

Hours Since Cutting.	Halves.		Thirds.		
	Apical.	Basal.	Apical.	Middle.	Basal.
29	1	0	1	0	0
30	3	0	1	0	0
31	6	1	1	0	0
32.5	9	2	3	0	0
33.5	12	4	6	0	0
34.5	14	9	8	0	0
35.5	17	11	9	2	0
37	18	14	10	3	0
39	19	16	10	6	0
41.5		18	10	6	0
43		19	10	9	1
44.5			10	9	3
45.5			10	10	4
46.5			11	10	4
48.5				10	6
50.5				11	6
52.5					8
54.5					9
56.5					11

It thus appears that the physiological differences along the stem which are responsible for the differences in regeneration rate at different levels extend further down the stem in *T. marina* than in either of the Atlantic coast species. Banus ('18) had previously shown a lack of difference in rate of regeneration of middle and basal thirds in *T. crocea*. Differences in the growth habits of the species are in harmony with the experimental findings. In *T. marina* (see illustration in Child, '15, p. 90), there are no branches but the base of each stem runs along the substratum as a stolon for some distance, then turns vertically, and gives rise to a new hydranth at its tip. This method of growth indicates that the hydranth in *T. marina* dominates a considerable length of stem. The Atlantic coast species, on the other hand, branch freely, frequently at a relatively short distance from the terminal hydranth. This fact suggests that the dominance of the terminal hydranth does not extend very far proximally. Beyond this limit the axial differences along the stem would be slight or absent. It is probable that if shorter distal pieces of the stem of *T. crocea* were cut into thirds, a difference would be found in the regeneration rate of the middle and basal thirds. In both my and Banus' experiments, however, rather long stems were used.

3. *Relation of Rate of Regeneration to the Length of the Piece.*—In a previous paper ('20) I reported that the length of the piece has little effect on the rate of regeneration when diameter and level of the distal cut are constant, except when the pieces are very short. This result was again verified on *T. crocea* and on the Maine species. Thus in 16 pairs of pieces of *T. crocea*, the two of each pair being 10 and 5 mm. long, respectively, with the distal cut taken at the same level in both, the 5 mm. pieces regenerated oral hydranths first in eight cases, the 10 mm. first in eight cases. It is understood that the diameter of the apical end was the same in the two members of each pair. At Swan's Island an experiment was performed comparing the rates of regeneration of pieces 15, 10 and 5 mm. long, the distal diameter and level of the distal cut being the same in the three pieces of each set. The rate of regeneration of the 5 and 10 mm. pieces was equal throughout this experiment; and this was also the case with most of the 15 mm. pieces, but a few of them preceded in production of hydranths the shorter pieces by a short time interval.

When the pieces are shorter than 5 mm. in length the production of hydranths is greatly slowed down, so that the regeneration of pieces 2 mm. in length, for instance, runs far behind that of longer pieces.

4. *Relation of Rate of Regeneration to the Diameter of the Stem.*—It was previously reported that the time between cutting and emergence of oral hydranths is noticeably shorter the more slender the stem. This was again verified on the Maine species. It is reasonable to believe that this result is correlated with the fact, given in the first part of this paper, that the rate of oxygen consumption is higher, the less the diameter of the stem.[1]

5. *Relation of Size of the Regenerated Oral Hydranth to the Length of the Stem.*—A number of measurements were made both at Woods Hole and Swan's Island of the size of the regenerated hydranths on stems of the same distal diameter and cut at the same distal level but of different lengths. Measurements were made with an ocular micrometer, and on the fully expanded hydranth some hours after emergence. The length of the tentacles, the length of the body (distance from tip of manubrium to constriction just below base of hydranth), and width of the widest part of the hydranth (part bearing the proximal tentacles) were measured. The most extensive experiment of this kind was done at Swan's Island. A number of pieces having the same apical level but of different lengths and diameters were cut and placed in one bowl. After regeneration the dimensions of the regenerated oral hydranths and of the original piece were taken. These data are given in Table III. and are arranged with reference to the diameter of the apical end of the piece.

These data show that the dimensions of regenerated oral hydranths are about the same on pieces of quite different lengths but of the same diameter and with their apical ends at the same level. This statement would probably not apply to very short pieces (under 2 mm.) but as shown in the table pieces as short as 2 mm. may regenerate hydranths as large as those from pieces several times as long. Some of the longest pieces may produce hydranths slightly larger than much shorter pieces but this difference is slight at best and in no wise proportional to the great differences in length of such pieces.

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[1] Stems whose diameter is smaller are of course also smaller in other particulars—total length and size of hydranths. As pointed out later they are simply younger as a rule than larger stems. It seems convenient to take the diameter of the stem as a measure of age. Wherever it is stated throughout this paper that the diameter of the piece is smaller it is to be understood that such pieces were taken from stems of general small proportions.

Table III.

Dimensions of Regenerated Oral Hydranths with Reference to Length of Piece.

Length in millimeters; other figures units of ocular micrometer.

Length of Piece, mm.	Diameter at Apical End of Piece.	Length of Body of Hydranth.	Width of Body of Hydranth.	Length of Tentacles.
2	6.0	15	6	12
5	6.0	18	8	11
9	6.0	21	10	15
2	6.5	14	8	10
5.5	6.5	20	9	12
5	6.5	15	7	12
15	6.5	24	11	17
15	6.5	20	8	14
2.5	6.8	19	8	14
5.5	6.8	22	9	14
5.5	6.8	20	7	10
10.5	6.8	23	12	18
17	6.8	23	12	15
2	7.0	20	7	16
5.5	7.0	18	8	12
5.5	7.0	19	8	13
6	7.0	19	9	14
10	7.0	22	9	14
10	7.0	23	10	15
10	7.0	25	11	15
10.5	7.0	19	9	14
11	7.0	18	8	12
5	7.2	19	8	11
15	7.2	24	10	13
5	7.5	19	10	15
5	7.5	20	9	12
5.5	7.5	20	9	12
11	7.5	20	10	15
11.5	7.5	24	12	22
16	7.5	27	12	19
2.5	8.0	21	8	15
10	8.0	25	12	17
2.5	8.5	22	9	11
5.5	8.5	24	12	12
5.5	8.5	22	9	12
10	8.5	22	11	16

6. *Relation of Size of the Regenerated Oral Hydranth to the Diameter of the Stem.*—Some experiments were performed on this matter. It was found that the size of the hydranth is slightly larger in stout than in slender stems when other factors are eliminated. This difference is detectable only when the difference in diameter of the stems is considerable. In Table III., where there are but small differences in diameter, no definite relation between dimensions of regenerated oral hydranths and diameter of the stems appears. But when stems differing markedly in diameter are compared the dimensions of the regenerated oral hydranths are seen to bear some slight relation to the diameter of the oral end of the pieces. One experiment of this kind is given in Table IV. Stout and slender stems from the same colony, of the same length, and cut at the same apical levels were allowed to regenerate and the resulting oral hydranths measured as in the preceding section. There were ten stems of each lot; these have been averaged in the table for brevity.

TABLE IV.

DIMENSIONS OF REGENERATED ORAL HYDRANTHS WITH REFERENCE TO THE DIAMETER OF THE APICAL END OF THE ORIGINAL STEMS.

Ten pieces in each lot, pieces 20 mm. long. Figures, units of the micrometer scale.

	Diameter Apical End.	Length Body of Hydranth.	Width Body of Hydranth.	Length Tentacles.
Lot of Slender Stems.				
Min.	5.5	19	8	15
Max.	7.0	26	11	21
Aver.	6.2	23	10	17
Lot of Stout Stems.				
Min.	7.5	22	11	18
Max.	11.0	30	15	22
Aver.	8.7	25	12	19

Table IV. shows that in the stouter stems the dimensions of the hydranth are slightly larger on the average than in the slender stems. The chief difference is in the width of the hydranth.

The result is readily understandable when it is recalled that in the regeneration of *Tubularia*, the new hydranth is laid down in the old cœnosarc. The new hydranth will then be necessarily broader the stouter the stem.

It will be perceived that the differences in dimensions of regenerated hydranths on stems of different diameter are in no wise proportional to the diameters. In Table IV., the diameters differ by 40 per cent. on the average, while the differences in dimensions of the regenerated hydranths average 10 to 20 per cent. Further, the more slender stems regenerate in a shorter length of time.

7. *Relation of the Size of the Regenerated Oral Hydranth to the Level of the Stem.*—In determining this matter it is necessary that the diameter of the apical ends of the pieces to be compared be the same, for, as shown above, diameter affects the dimensions of the regenerated hydranth. It is a little difficult to obtain pieces from different levels of the same apical diameter since in *Tubularia* the stem generally tapers towards the base. However, it is occasionally possible to find stretches of stem of approximately the same diameter throughout or even some which increase in diameter proximally. Only such have been used in making the comparison. From such stems apical and basal pieces of equal length were cut and after regeneration the dimensions of the regenerated oral hydranths determined. Some data of this kind, obtained at both Woods Hole and Swan's Island are given in Table V.

It is obvious to the eye and measurements also demonstrate that in pieces of the same diameter and length but taken from different levels, the dimensions of the regenerated oral hydranth are nearly always greater on the apical than on the basal piece. Level is thus the most important factor in determining the dimensions of regenerated oral hydranths. These size differences of oral hydranths also of course appear in pieces cut from the usual type of stem, where the diameter of the basal piece is smaller than that of the apical piece; and are too great to be accounted for merely on the differences in diameter.

TABLE V.

DIMENSIONS OF REGENERATED ORAL HYDRANTHS ON PIECES OF THE SAME LENGTH AND DISTAL DIAMETERS BUT FROM DIFFERENT LEVELS.

Figures, units of the micrometer scale.

Level of Pieces.	Diameter Distal End.	Length Body of Hydranth.	Width Body of Hydranth.	Length Tentacles.
Apical.	10.0	15	7	20
Basal.	10.0	12	7	16
Apical.	5.0	14	5	15
Basal.	5.0	10	2	7
Apical.	6.5	20	10	23
Basal.	6.5	16	7	18
Apical.	5.5	30	9	23
Basal.	5.5	17	8	27
Apical.	7.1	20	10	25
Basal.	7.1	17	9	18
Apical.	6.6	27	11	45
Basal.	6.8	24	11	45
Apical.	6.4	28	10	40
Basal.	6.4	23	10	35
Apical.	6.3	23	7	27
Basal.	6.3	21	7	12

DISCUSSION.

The foregoing facts together with others available from the literature support the conception of the existence of a metabolic gradient along the main axis in *Tubularia* and other lower forms; and of a relation between this gradient and the rate of regeneration.

It is shown in this paper that the rate of oxygen consumption in the stem of *Tubularia* is higher in apical than in basal levels. It thus appears that there exists a gradation in respiratory rate along the stem of *Tubularia*. Similar respiratory gradients along the main axis were previously reported for other lower invertebrates: *Corymorpha*, *Grantia*, *Planaria*, several annelids (Hyman, '22, '23, '25, Hyman and Galigher, '21). It is reasonable to believe that they are of universal occurrence among at least the lower Metazoa. We believe that such differences in rate of chemical activity (with which are doubtless associated other graded differences) constitute the basis of the phenomenon of polarity.

It appears further that there exist in these organisms permanent electric currents whose direction of flow bears a definite relation to the respiratory gradient (for data on *Tubularia*, see Hyman, '20, for other hydroids, Hyman and Bellamy, '22, Lund, '22).[2] This relation is the following: any part of the organism is electronegative (in the external circuit) to any part having a lower respiratory rate than itself. It is probable that the gradation in rate of chemical activity is the chief cause of the electrical gradient. Some biologists are of the opinion that these electric currents constitute a tool, so to speak, which enables one part of an organism to affect another part.

There is some indication that in the hydroids the gradient is steeper in the more apical levels and gradually flattens out basally. This inference is drawn chiefly from the electrical data, the potential difference being greatest in apical levels (Hyman and Bellamy, '22, pp. 332–33, Lund, '22, p. 490); but in *Corymorpha* the respiratory evidence is to the same effect (Hyman, '22). Regardless of the slope of the gradient in distal levels, it appears certain from electrical and other data that the gradient is slight or absent or even reversed in the proximal levels of hydroid stems (see references just given). It follows that at a certain distance from the apical end, the primary gradation practically disappears and new gradations running in the same or the reverse direction may be initiated. The distance to which the primary gradation extends coincides with the limits of the individual and beyond this point buds, zooids, etc., may arise, if the constitution of the protoplasm permits asexual reproduction; or if asexual reproduction is impossible, the basal or caudal parts of the organism may be more or less independent physiologically or nervously of anterior levels. The existence of an oral or apical end inhibits the formation of any other oral or apical end within the distance limit over which the control of the former extends (see Child, '15, Chapter IV.).

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[2] In a later paper ('25) Lund reversed his statement in the 1922 paper as to the direction of the current in *Obelia*, without offering any explanation of the contradiction. It may be pointed out that Hyman and Bellamy ('22) tested the P. D. along the main axis of colonies of a species of *Obelia* common at Friday Harbor and identified by Professor Nutting as *Obelia borealis* and of *Obelia geniculata* at Woods Hole; and in both species found distal levels electronegative to proximal in agreement with the statement in Lund's 1922 paper but contrary to his statement in 1925.

Differences in rate of regeneration (time between cutting and completion of oral or apical structures) with respect to level constitute further evidence of the existence of a metabolic gradient in hydroids. The more apical the level in the whole within the limits of the primary gradient from which the piece is taken the more rapidly does it produce a new apical end. This generalization has been shown to hold in a large number of lower invertebrates, mostly cœlenterates, *e.g.*,—*Eudendrium* (Goldfarb, '07), *Tubularia* (Driesch, Morgan, Child, etc., for references see Hyman, '20), *Corymorpha* (Torrey, '10), *Pennaria* (Gast and Godlewski, '03), *Obelia* (Billard, '04, Lund, '23), *Cerianthus* (Child, '03), *Planaria* (Child, '11), annelids (Hyman, '16). Further proof of the correctness of this generalization with regard to *Tubularia* is presented in this paper. It is also shown that such axial differences in rate of regeneration are independent of size or mass differences at different levels. It can scarcely be doubted that the metabolic gradient is the direct or indirect cause of the apico-basal gradation in rate of regeneration of oral or apical structures.

Another instance of the dependence of regeneration rate on metabolic rate is the difference in these regards between organisms of different ages (sizes). It is shown in this paper and previously (Hyman, '20) that in *Tubularia* the rate of regeneration is more rapid the smaller the diameter of the piece; and further that the rate of oxygen consumption is higher the smaller the diameter. There is thus a correlation between rate of production of oral hydranths and rate of respiratory metabolism; and it is scarcely to be doubted that the latter is the direct cause of the former. It is probable that in *Tubularia* the dimensions of the stem (diameter, total length, size of hydranth) vary inversely with age and that we are really dealing here with age and not size differences. An inverse relation between respiratory rate and age (size) appears to be universal throughout the animal kingdom. In a previous paper (Hyman, '19) I reviewed this matter and quoted a considerable body of evidence in support of this generalization. Since then I have obtained additional evidence (unpublished) of the inverse relation between respiratory rate and size in *Corymorpha*, starfish, nudibranchs, and tadpoles; and other data have appeared in the literature (*e.g.*, Smith, '25). The relation here found in *Tubularia* between rate of regeneration and age probably also is of general application. Przibram ('07) gives a discussion of this matter and reaches the generalization that the rate of regeneration is more rapid the younger the animal and declines with age.

Not only is there a relation between rate of regeneration (time between cutting and completion of oral or apical structures) and respiratory rate but the amount of tissue regenerated in a given time appears also to be dependent upon metabolic rate. This applies both to axial differences and age differences. In general the more apical the level from which the piece is taken the larger is the size of the oral or apical end regenerated, and the greater the total mass of regenerated tissue. This result cannot be ascribed to differences in mass of pieces from different levels for it also holds when the pieces are of equal mass. It is shown in this paper that in *Tubularia*, the regenerated oral hydranth is larger on apical than basal pieces, when the length and apical diameters of such pieces are the same. Driesch ('99) and Child ('07) had previously noted that the length of the primordium of the oral hydranth is greater the more apical the piece. A similar relation between level and the size of regenerated apical structures or total amount of tissue regenerated was observed by Billard ('04) in *Obelia*, Child ('03) in *Cerianthus* and *Planaria* ('11) and Morgulis ('07) in annelids.

The amount of tissue regenerated in a given time is also greater the young (smaller) the organism, relative to its size. This is shown to be the case in *Tubularia*, pieces from smaller stems producing relatively larger hydranths in a shorter time than pieces of equal length from large stems. A similar relation between age and rate of formation of new tissue was found by Zeleny ('07), Ellis ('08), and Scott ('09).

A third factor appears to be involved as regards the amount of tissue regenerated in a given time. This factor is the degree of injury relative to the mass of the regenerating piece or organism. Some years ago under the leadership of Zeleny there was considerable interest in this matter. A number of papers dealing with this subject were published (Zeleny, '03, '05*a*, '05*b*, '07, Ellis, '07). A general agreement was reached by the workers in this field that any one part is replaced at a more rapid rate, the greater the amount of tissue removed at the original operation. It is possible that this result also depends on metabolic rate; for every wound is the locus of an increase in metabolic rate and the greater the number of wounds and the smaller the mass of tissue remaining the greater is the stimulation of respiratory metabolism not only at the wounds but also in the adjacent uninjured parts.

In general, then, it appears that the size of regenerated oral or apical structures and the amount of tissue produced in a given time are causally related to the metabolic rate of the regenerating mass.

When metabolic factors such as level, age, or wound stimulation are not involved, the mass of the piece appears to have little or no effect upon the amount of tissue regenerated in a given time. Thus it is shown in this paper for *Tubularia* that pieces differing considerably in length and hence total mass regenerate oral hydranths of equal size in equal lengths of time. Because of this lack of relation between mass and regeneration it can be stated as a generalization that the smaller the original mass (within certain limits of course) the greater relative to its mass is the amount of tissue produced in a given time. Other data in support of this statement will be found in many of the papers already cited.

It remains to consider a paper by Lund ('23) on regeneration in *Obelia*, in which paper certain conclusions are stated which seem to be at variance with those presented here. In the regeneration of *Obelia* as previously noted by Billard ('04) a process grows out from the cut surface and the hydranth differentiates at the end of this outgrowth. Lund has studied the time occupied by this growth process and the rate of elongation of the outgrowth in a series of pieces cut in apico-basal sequence from the main stem of colonies of *Obelia*. Lund finds as did Billard

('04) that the time between cutting and completion of the polyp is shorter the more apical the piece. But according to Lund the time between the beginning and end of the outgrowth is the same at all levels. By defining the regeneration period as the time period during which the outgrowth is elongating, Lund is able to reach the conclusion that the "rate of regeneration" is the same at all levels. It may merely be pointed out that adopting some particular definition in no wise alters the facts of the matter, which are the same for *Obelia* as for other cœlenterates. Lund is able to state that the rate of regeneration does not differ at different levels only because his definition of the expression "rate of regeneration" differs from that used by other workers. The point raised by Lund that the time interval from the beginning to the end of the elongation process is the same at all levels may be correct but it does not seem to me to be proved by his tables and graphs. However, it is difficult to come to any decision on the matter, as neither the time of beginning of growth of the apical pieces nor of completion of growth of the basal pieces is given. It is very probable, nevertheless, that the chief differences in regeneration rate at different levels lie in the early part of the regeneration period. It is admitted by Lund and is shown by his tables and graphs that the rate of elongation of the outgrowth is faster the more apical the level. Billard ('04) had previously made a similar observation; he noted that the sum of the lengths of the outgrowth at both ends of each piece is greater the more apical the piece. In the face of his own data, Lund still attempts to maintain the conclusion that the rate of elongation is the same at all levels on the assumption that the rate of elongation decreases apico-basally because the mass of the pieces decreases in the same direction. He assumes without any proof whatever that the amount of tissue regenerated is proportional to the mass of the piece. Such an assumption is incorrect. As already pointed out considerable differences in length of pieces have no effect on either the time required for regeneration or the amount of tissue produced in that time; and such slight differences in amount regenerated as are correlated with differences in diameter are counterbalanced by the more rapid rate of regeneration of pieces of smaller diameter. All of the available evidence indicates that the mass of regenerated tissue is not proportional to the original mass of the piece but to the contrary is relatively greater the smaller the latter. In view of all of the facts it is practically certain that the apico-basal sequence in rate of elongation observed in *Obelia* pieces is the result of differences in level. Even though the time occupied by the elongation process may be the same at different levels, as insisted by Lund, still the length of outgrowth produced in that time is greater the the more apical the piece; and hence the "rate of regeneration," even using this expression as defined by Lund, decreases apico-basally in *Obelia* as in other lower forms.

SUMMARY.

1. The rate of oxygen consumption per unit volume of cœnosarc is greater in apical than in basal halves of distal regions of the stem of *Tubularia*.

2. The rate of oxygen consumption per unit volume of cœnosarc is greater the younger the stem (smaller its diameter).

3. The time between cutting and completion of oral hydranths is shorter the more apical the piece in pieces of equal length from distal levels of the stem of *Tubularia*.

4. The statement in 3 may or may not hold for proximal regions of the stem, depending on the species.

5. The time between cutting and completion of oral hydranths is independent of the length of the piece when the apical end of the pieces is taken at the same level, except in very short pieces.

6. The time between cutting and completion of oral hydranths is shorter the younger the stem (smaller the diameter).

7. The size of the regenerated oral hydranths is almost entirely independent of the length of the piece, when the apical end of the pieces is taken at the same level, except in very short pieces.

8. The size of the regenerated oral hydranth is slightly smaller especially as to width the smaller the diameter of the stem but not proportionally smaller.

9. The size of the regenerated oral hydranth is absolutely larger on apical than on basal pieces of equal mass taken from distal regions of the stem.

10. In general there is a relation between respiratory rate and

regeneration. The higher the respiratory rate the shorter is the time interval between cutting and completion of oral hydranths and the larger is the size relatively or absolutely of the regenerated oral hydranth.

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THE VALUE OF INSTINCT AS A TAXONOMIC CHARACTER IN SPIDERS.

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The study of animal behavior under experimental conditions has brought into sharp opposition the supporters of the idea of the rigidity of instinct with the defenders of its plasticity. The inability of the animal, especially of invertebrates to meet new and unaccustomed conditions in human fashion has furnished the chief argument in favor of the attitude of the modern school of behaviorists. The interpretation of an animal as a machine activated by stimuli to reflexes with an outward appearance of intelligence, yet without intelligent control of its actions has been and still is sufficiently often discussed by both parties not to need further discussion here. Some day I hope to put together for print some observations on the plasticity of instinct in spiders, which I have made gradually during the past years. For the present I want to content myself with another aspect of the problem.

The discussion referred to above naturally centers round the phenomena of *individual* behavior. Ascending from individual to species and widening our observations to different geographical regions and environmental conditions, influenced by our preference for the one or the other school of behaviorism we lay stress either on the individual modifications or the specific similitudes of instinct. The same applies of course to generic and family characteristics. The instincts of spiders are apparently particularly rigid in this respect and easily traceable to family relationship in burrowing habits, snare construction, courtship, mating and care of cocoon. Thus all representatives of the family Argiopidæ build orbwebs, all Lycosidæ carry the cocoon attached to their spinnerets, etc. In fact in the earlier days of arachnology the habits were used for family or larger group distinction and spiders were divided into Terricolæ, Tubicolæ, Citigradæ, Saltigradæ, Laterigradæ, Retithelæ, and Orbithelæ. It was soon recognized, however, that orbwebs are constructed by some spiders anatomically very different from Argiopidæ and since separated from the latter into a family Uloboridæ among the supposedly more primitive division of Cribellatæ. The same was done for the Dictynidæ among the Cribellatæ, which were originally placed with the Retithelæ. The similarity in web construction was explained by the assumption of parallelism in both groups. Similar though not such pronounced parallelism was found in the case of various Tarantulæ or Theraphosid spiders, the habits of which are comparable with those in true spiders. Parallelism of such kind may be easily explained as adaptive to similar conditions of life. But it would be difficult to conceive the construction of such a complicated and perfect snare as an orbweb as a manifestation of habits acquired independently by every genus of Argiopid spiders. The general features of this habit must have been acquired before the splitting up of the original stock into new species and persist notwithstanding the fact that the family spread all over the world from the tropical rain forests to the cold zones of the North and South, and comprises an enormous number of species as unlike each other in size and appearance as *Nephila* with its inch and a half long body and four-inch spread of legs and *Theridiosoma* scarcely a tenth of an inch in length, *Gasteracantha* with her abdomen much wider than long and adorned in some species with long, curved spines exceeding many times the length of the body and *Tetragnatha* with her soft abdomen often many times longer than wide and legs so drawn out and kept in a peculiar fashion that the spider in its web has the appearance of a little twig. All these modifications of structure must have therefore appeared later and influenced only the subordinate features of the habit, the details of the general plan of structure of the geometric web. It was this among other considerations that inclined me to the view which I still hold that the Argiopidæ are descendants of Uloboridæ.

The manner in which the female takes care of the cocoon with eggs is another habit characteristic of whole families. In fact so distinctive is this habit in some families that on the strength of it the great French arachnologist Simon placed the genus *Rhoicinus* among the Lycosidæ, although neither the disposition of the eyes, nor other important morphological structures are of the type to warrant the inclusion of the former in the very characteristic family of ground spiders. Says Simon in his "Histoire Naturelle des Araignées": "Les quelques espèces, pour lesquelles j'ai proposé le genre *Rhoicinus*, sont très anormales pour la famille des *Lycosides*, dont elles n'ont pas la disposition oculaire; si l'on ne tenait compte que de ce caractère, on les rapprocherait des *Cybaeinæ*, particulièrement des *Campostichomma*, mais leurs trochanters sont entaillés d'une profonde échancrure apicale, leur griffe impaire ne porte q'une seule petite dent basale *et, de plus, j'ai surpris l'une des espèces portant aux filierès son cocon globuleux, comme l'aurait fait un Lycosa*." (Italics are mine, A. P.) Without discussing the case in question since our knowledge of *Rhoicinus* is as yet very imperfect and I have no first-hand acquaintance with the genus, I merely wish to point out that of the characters mentioned by Simon the notch in the trochanters is a character occurring in other families besides the Lycosidæ, such as Pisauridæ, Clubionidæ, Argiopidæ, etc., the number of teeth in claws is very variable, changing in other families from genus to genus, while the disposition of the eyes is undoubtedly a very old character and remarkably persistent within families, being at the same time very little affected by age. Thus the deciding character in this case remains the cocoon-carrying habit and the question arises, which character is more subject to modification, a fundamental, old morphological character or a certain habit?

On one of my excursions in Porto Rico I was fortunate enough to make an observation which proves definitely that at least the cocoon-carrying habit may be acquired by spiders the morphological characters of which prevent their inclusion in the family Lycosidæ beyond any possibility of dispute. While turning over a rock in a field near Aguas Buenas I noticed two little spiders on the underside of the rock, carrying their cocoons Lycosa-fashion, attached to the spinnerets. Both proved to be new species to which I have given respectively the names of *Bathy-*

phantes ovigerus and *Lithyphantes oöphorus*. The detailed descriptions of the species will be given later in another place, in my study of Porto Rican spiders. *Bathyphanthes* is a well-known genus of the family Linyphiidæ and has numerous representatives in the United States, Europe and other countries. *Lithyphantes* belongs to the family Theridiidæ and has also a wide distribution. Both are small spiders, *B. ovigerus* measuring 2.87 mm. in length, *L. oöphorus* 1.628 mm. Of the former species I have collected later several females and a male under similar conditions not far from Rio Piedras, of the *Lithyphantes* a second female under a cocoanut shell on the beach at Puerto Nuevo Point. Thus I was enabled to make some observations on both species under laboratory conditions. Both behaved much in the same manner.

The cocoon of *B. ovigerus* is globular and rather large in proportion to the size of the spider, being fully 2 mm. in diameter, with thin, white walls and a few eggs well visible through the silk. The spider makes no web of any kind, but walks dragging the cocoon behind her and if disturbed runs for shelter. Deprived of the cocoon the spider shows signs of uneasiness and on discovering the cocoon grasps it with her cheliceræ, bends her almost globular abdomen until the spinnerets reach the cocoon from below, releases her hold on it with the cheliceræ and starts off dragging now the cocoon behind her. For two days she behaved this way. On the third day I was surprised to find her sitting on the bottom of the jar while the cocoon was hanging close by, suspended by a few threads in a small web made of loose threads and much of the type common in small Linyphiids. The explanation of the change in behavior was furnished the same day when minute spiderlings emerged from the cocoon. Toward the end, then, of her maternal duties the original instinct common to all Linyphiids asserted itself, showing that the species still retains some of the family habits and that the new habit did not develop to the point characteristic for Lycosids, where the spiderlings are carried by the mother on her back until they have moulted and are large enough to shift for themselves.

Lithyphantes oöphorus has an elongated abdomen vividly marked with black and white, the colors forming a pattern of a type more or less common for the genus. The cocoon is globular, still more out of proportion with the size of the spider, being 1.2 mm. in diameter, with thin, white walls barely covering the dozen eggs. The cocoon was firmly attached to the spinnerets and the spider would not release it when disturbed. On the second day, however, she was found sitting on the bottom of the jar while the cocoon was suspended by a few losely woven threads. On examination, it was found that two of the eggs developed into spiderlings, while the remainder were parasitized by an insect. Since the spiderlings emerged from the eggs there is nothing singular in the fact that the mother abandoned the cocoon.

Here, then, we have proof positive that one of the most stable instincts in spiders may be modified and what is still more interesting may be modified in the same sense in two different families, presenting a clear case of adaptive parallelism. It is true that the two families are closely related. In various instances species placed in the family Linyphiidæ were later transferred to the family Theridiidæ, when the presence of the "comb" on the fourth tarsi was ascertained. In the great majority of species the distinction between the two families is quite pronounced and certainly there is no close relationship between the genera *Bathyphantes* and *Lithyphantes*.

It is more difficult to decide which of the two methods of taking care of the cocoon should be considered the more primitive one. At first thought it would seem as if carrying the cocoon attached to the spinnerets were simpler than hanging it up in a web. The spinnerets and the cheliceræ are the organs employed in the making of the cocoon. The former produce and weave the silk, the latter are used in clipping the sheet and in joining the seam. We know spiders which carry the cocoon in their cheliceræ, as for example, *Spermophora* and *Scytodes*. The families to which these two genera belong have been often considered to be primitive. It seems to me however, that the family Pholcidæ to which *Spermophora* belongs, shows all signs of specialization, especially in the structure of the cephalothorax and legs. At any rate, we know as yet no truly primitive spiders which would carry their cocoon attached to the spinnerets, and the Lycosidæ which generally possess this habit, are not in any sense primitive. For this reason it cannot be possibly maintained that *B. ovigerus* and *L. oöphorus* have simply dropped the habit characteristic of their respective families and reverted to a more generalized and fundamental habit original with all spiders. The conclusion is inevitable that in the course of evolution, before any morphologically considerable changes have occurred, our two species of spiders have modified their habit.

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BIOLOGICAL BULLETIN

FURTHER STUDIES ON THE LIFE HISTORY OF *CRASPEDACUSTA RYDERI*, A FRESH-WATER HYDROMEDUSAN.

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In 1924 I published an account of a study of *Craspedacusta*. This study included the morphology of the medusa and hydroid and the development of hydroids and medusæ from buds. The life history was followed as completely as possible. For these details, the reader is referred to my former paper (Payne, '24). The story remained incomplete as all the medusæ were females. Since the publication of the paper I have continued to make observations in Boss Lake, but have nothing new to add except that medusæ were not found during the summers of 1924 and 1925. The hydroids were still present in 1924, but no efforts were made to find them in 1925. Since the hydroids were present, at least in 1924, conditions must have been unfavorable for medusæ development. Males of *Craspedacusta* were reported by Hargitt ('08) and Garman ('16 and '24). I have been anxious to get the two sexes together and complete the life history. The method which suggested itself was to find the hydroid of one of these male strains and transplant it to Boss Lake, Elkhart. The medusæ live only a short time out of their native habitat, so to transplant them would be impossible. The hydroid, on the other hand, is very hardy. A change of water does not affect it. The main requirement is a food supply.

Through the kindness of Professor Garman, I learned where the medusæ had been most abundant in Benson Creek, Kentucky. This gave me a good idea where to look for the hydroid. As noted in *Science* ('25), I made a trip to Benson Creek July 30 and found a few hydroids on the flat stones in shallow water. There had been much rain during the summer in this region and the creek was muddy and most of the rocks were covered with slime. The hydroids were small and were not producing buds. Three weeks later the water had cleared and some of the slime had been washed from the rocks in swift water. Hydroids were found in abundance and transplanted to Boss Lake. They were still small and were not reproducing. No medusæ were found in the Creek. I am inclined to think that no medusæ buds were formed, due to the many rains, the muddy water and slime, and the small supply of food.

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[1] Contribution No. 211.

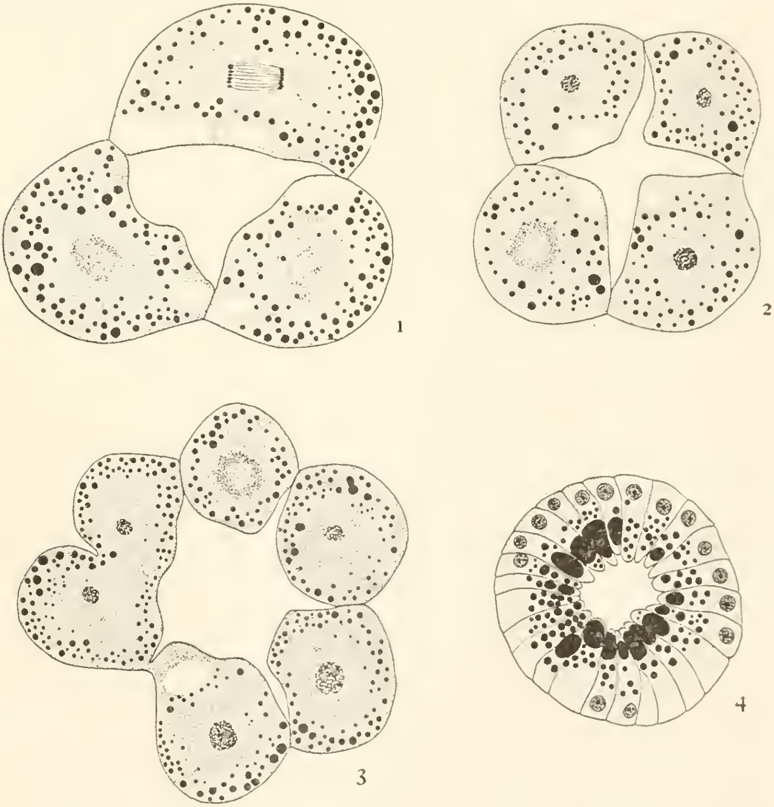
September 11, Dr. W. R. Allen, of the University of Kentucky, wired that medusæ were reported in the Kentucky River. The next day he and I visited the river near College Hill, about 40 miles southeast of Lexington. We found medusæ in abundance at the first place we reached the river, and had no difficulty in collecting several hundred during the course of an hour by dipping them up one at a time. This is not the first time that medusæ have been seen in the Kentucky River. I have learned, with the assistance of Dr. Allen, that Mr. Leonard Giovannoli, now a graduate student in zoölogy at the University of Kentucky, saw them at High Bridge in 1917. This was only one year after Garman's discovery in Benson Creek. High Bridge is 50 miles up the river from the outlet of Benson Creek into the Kentucky River. In 1922 medusæ were seen by Mrs. Alberta W. Server, at Valley View, 35 miles still further upstream. During the past summer, Mr. Wiley Sams, in the course of a canoe trip up the river, saw medusæ at three widely separated places. The uppermost point was near College Hill, which is 40 miles farther upstream than Valley View. Mr. Sams reported the medusæ as very numerous.

As Garman had reported males from Benson Creek and since both sexes had never been found in the same place, I took it for granted that the medusæ we collected were males. Upon arrival in Bloomington with my catch I made an examination of the medusæ and also the eggs which had been shed in the water.

To my surprise I had both sexes and many developing eggs. A week later a second collection from the same place was made by Mr. Giovannoli and brought to Bloomington. They were collected and brought to Bloomington the same day, hence they reached me in good condition. From this lot I was able to work out most of the essential stages in the development of the egg into the hydroid.

Development.

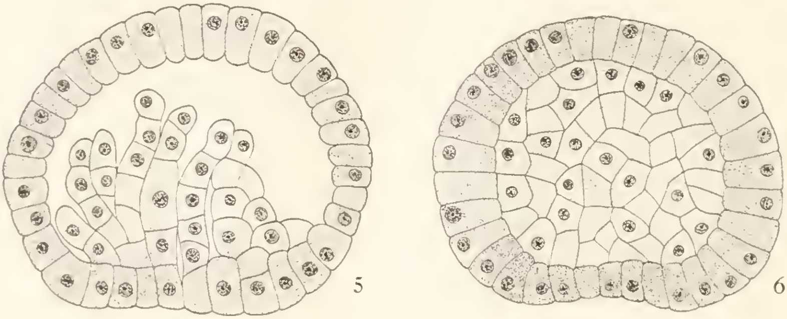
No attempt was made to make a detailed study of the developmental stages, but sufficient work was done to give the main trend of events.



Figs. 1, 2, 3, and 4. Sections of some early cleavage stages; 4, a ciliated blastula.

The germ cells lie in the ectoderm of the gonads, which hang as sack-like pouches from the radial canals. In the ovaries the eggs form a single layer with small cells wedged in between. In the testes there are many cells showing all stages of development from the spermatogonia to the mature sperm. The first polar spindle is formed before the eggs leave the ovary, but I have never found the division completed. In material from Boss Lake the late anaphase has been seen in a few instances (Payne, '24), but many eggs, in material from Boss Lake and also from Kentucky, show the first polar spindle in metaphase or slightly earlier.

The first cleavage is equal, but after this inequalities enter (Figs. 1, 2, and 3). The rate of cleavage becomes unequal from the two-cell stage. Figure 1 shows a three-cell stage. Even at this time a large cavity is present between the blastomeres. Continued cleavage results in the production of a blastula, the cells of which are long, pointed at the inner ends, and larger at the peripheral ends. The cavity of the blastula is small (Fig. 4). The yolk has accumulated near the inner ends of the cells, so that even in the living blastula the outer ends look clear while the inner ends are opaque. The nucleus lies in the outer clear end. The larva is ciliated and swims about freely at this stage. At what stage the cilia disappear I am not sure, but it occurs before elongation begins.



FIGS. 5 AND 6. 5 shows the method of endoderm formation; 6 the solid endoderm.

The method of endoderm formation is similar to, but shows some variations from the process in other Hydromedusæ. There is a slight invagination at one side, probably the vegetal pole.[1]

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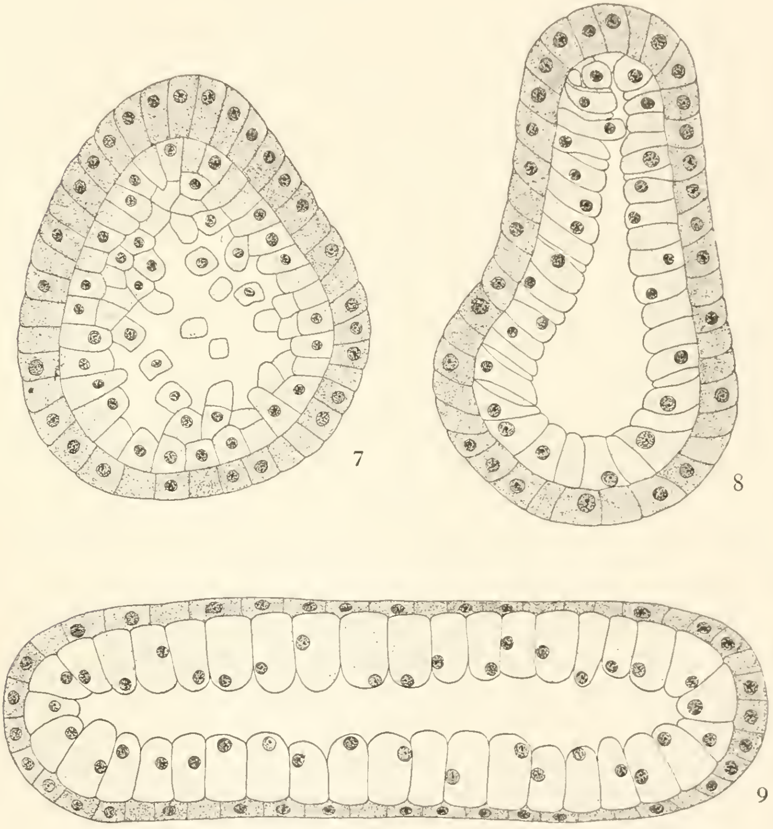
[1] I shall use the terms "vegetal" and "animal" pole to describe the differences observed rather than to indicate a distinct polarity. I have not oriented the egg to make sure that endoderm formation occurs at the vegetal pole.

At the same time the cells at the place of invagination divide and migrate into the segmentation cavity (Fig. 5). From the figure it may be noted that the cells extend into the cavity in the form of strands. This division and migration continues until the segmentation cavity is filled with cells (Fig. 6). The cells of the outer ectodermal layer are still elongate but less so at the vegetal pole (Fig. 6). This difference between the length of the cells persists throughout the later stages of development. It is



FIGS. 7, 8, AND 9. 7 shows the beginning of elongation and the formation of the gastrovascular cavity; 8, a continuation of the same processes; 9, the endoderm is now a single layer of cells and the gastrovascular cavity is formed. This stage now attaches at one end. The mouth opening and nematocysts form, thus completing the hydroid development.

All figures drawn to the same scale.

the only method of distinguishing the two ends of the young hydroid (Figs. 7, 8, and 9). Shortly after the formation of the solid endodermal mass of cells, the whole structure begins to change shape. Elongation begins at the animal pole (Fig. 7). This produces a larva somewhat pointed at one end and large at the other. About this same time a cavity begins to form in the endodermal cells (Fig. 7). Presumably this is formed by migration and rearrangement of cells. Elongation continues and along with it the cavity in the endoderm enlarges (Fig. 8). At the end of these processes the larva is practically uniform in diameter from end to end and has a single layer of endodermal cells surrounding a central gastrovascular cavity (Fig. 9). The one distinguishing mark is the unequal size of the ectodermal cells at the two ends. Which of these stages is the typical planula is difficult to say. Dalyell's planula was elongated and ciliated, and the endoderm was still a solid mass. In *Craspedacusta* the cilia are lost and the endoderm is formed before elongation begins. The gastrovascular cavity is formed during elongation. This elongated larva or planula attaches at one end and grows into the typical hydroid. A description of the hydroid need not be given as this was done in my former paper (Payne, '24).

This completes the life history of this interesting form, which for several years has made sporadic appearances here and there, only to disappear without revealing to us much of its story.

SEX.

In all earlier finds of *Craspedacusta*, with the exception of those in Boss Lake, the medusæ were reported to be males. In Boss Lake all medusæ were females. Why were both sexes not found together? In my former paper I answered this question by suggesting that possibly the environment might be a determining factor; also that the hydroids might be male or female producing. It was my inclination to favor the latter interpretation. The fact that we now find both males and females in the same environment would also favor such an interpretation. The sexes are approximately equal in number in the Kentucky River, a count of 110 giving 52 females and 58 males. They are also equal in size in so far as I can judge with the eye. The only method of distinguishing them is by an examination of the gonads.

Distribution.

In my former paper I stated my belief that *Microhydra* and *Craspedacusta* were merely hydroid and medusa stages of one and the same species, namely *Craspedacusta ryderi.* I also stated that I believed the hydroid much more widely distributed than formerly thought, and that it was only when conditions were favorable that the medusæ appeared. My further studies have strengthened this point of view. No medusæ appeared in Boss Lake in 1924 and 1925. I know the hydroids were present in 1924. No medusæ were present in Benson Creek in 1925. Yet I found the hydroids.

The fact that both males and females occur in the Kentucky River may indicate that this is their point of origin from a marine life. During the elevation of this region, which at times has been a part of the sea, it is possible that *Craspedacusta* became cut off from the main sea and that it was able to adjust itself in the slow change from salt to fresh water.

Size.

The first medusæ taken in Boss Lake, September 1919, were large, measuring from one half to three fourths of an inch in diameter. They were perfect specimens. In later summers they were never so large nor so perfect. I attributed this change to the presence and destructive action of many amœbæ. The medusæ taken in the Kentucky River were large and perfect, similar to the first specimens taken in Boss Lake. I did find amœbæ on them, however, when they were allowed to live in the laboratory until they showed signs of disintegration.

Systematic Position.

The systematic position of *Craspedacusta* has been a debatable one. This is not surprising, however, when we look at the taxonomic work on the Hydromedusæ. A glance at the literature tells one that classification in many cases has been attempted without knowing much or anything of the life history. Hydroids have been classified independently of medusæ and medusæ independently of hydroids. Originally such studies were necessary, no doubt, but I see no reason why they should continue. Neither do I see how we can arrive at a satisfactory classification until we know the complete life-history of each form studied. This may seem a rather discouraging viewpoint, since we now know the life-history of so few, and when we consider the enormous amount of work and the difficulties involved.

Mayer ('10), in his classification of the medusæ of the world, describes the Leptomedusæ as follows: gonads on the radial canals; otoliths, if present, of ectodermal origin; medusæ arise through alternation of generations from Campanularian hydroids. He describes the Trachymedusæ as medusæ having marginal vela, uncleft bell margins, and lithocyst concretions of endodermal origin. In all textbooks we find statements that Trachymedusæ are without a hydroid stage.

Where then does *Craspedacusta ryderi* belong in our present system of classification? Lankaster ('81), Douglas ('12), Mayer ('10), and others have placed *Craspedacusta* among the Trachymedusæ. Allman ('80) placed it among the Leptomedusæ. On the other hand Günther ('94) described it as a medusa descended from Leptomedusan ancesters, which had developed sense-organs with an endodermal axis independently of the Trachymedusæ. Mayer ('10) regards the Trachymedusæ as transformed actinulæ. He says: "They (Trachymedusæ) commonly develop through an actinula larva in which the bell grows out as a collar-like, or intertentacular lappeted expansion from the sides of the body after the tentacles have appeared, and the tentacles of the actinula become those of the medusæ. The medusa of the Leptomedusæ is formed upon a different plan, for the tentacles grow outward from the bell-margin after the bell has developed. I believe, therefore, that the bell of the Trachymedusæ is not homologous with that of the Leptomedusæ. It is evident that the endodermal otoliths of the Trachymedusæ are not homologous with the ectodermal otoliths of Leptomedusæ. I believe that the medusa-shape has been acquired independently in the *Trachylina* and *Leptolina* forms of veiled medusæ."

Microhydra, along with *Protohydra* and others, has either been placed in a separate subdivision of the Hydromedusæ, or with the Tubularian hydroids.

Since we now know the life-history, we are better able to discuss the systematic position of *Craspedacusta*. First, we find a complete alternation of generations. By this I mean that we have hydroids which give rise to medusæ by means of buds, an asexual process, and medusæ, which, by a sexual process, give rise to the hydroids. If Mayer is correct, it is evident that the medusa of *Craspedacusta* is Leptomedusan in structure and in development, with the exception of the origin of the otoliths. Most certainly it is not a transformed actinula. It arises as a bud from the hydroid. The bell is formed as in the Leptomedusæ and the tentacles are outgrowths from the margin of the bell. The medusa is a typical completely formed medusa when it breaks away from the hydroid. For the details of this process, see my former paper (Payne, '24). I do not see how the hydroid of *Craspedacusta* can be interpreted in any other way than as a hydroid. There are no tentacles, but in other respects it is a typical hydroid and behaves as such. Is it a Tubularian or a Campanularian hydroid? The only essential difference between the two groups is the presence of hydro- and gonothecæ in Campanularia and their absence in Tubularia. The basal half or two thirds of the hydroid of *Craspedacusta* is surrounded by a covering which is in part a secretion, but whether it could be called a hydrotheca and the hydroid classed with the Campanularia is doubtful. If the covering is not a hydrotheca, perhaps the hydroid could be classed as a Tubularian. According to the present classifications there is no such thing as a Trachymedusan hydroid.

The medusa of *Craspedacusta*, with the exception of the endodermal origin of the otoliths, is Leptomedusan. This characteristic is Trachymedusan, but in so far as I can judge, it is the only exclusive Trachymedusan characteristic which the medusa has. Where then, should *Craspedacusta* be placed? I do not see how we can place it in any of the existing groups as these groups are now defined. It would seem to me that it is more closely related to the Leptomedusæ, however. While I do not care to place *Craspedacusta* in either of these groups as they are now, neither do I care to create a new position for it. This must wait for more extensive studies upon the life histories of the Hydromedusæ. When these are completed we shall be in a better position to make a new classification.

Another structure to be reckoned with in a new classification is the presence of an ocellus-like organ in *Craspedacusta*. It lies at the junction of the outer margin of the bell and velar ectoderm and near the nerve cord. This sense organ (probably tactile in function) is ectodermal in origin (see Payne, '24).

In connection with the taxonomic discussion of *Craspedacusta* it is of interest to note that Joseph ('25) has been able to complete the life history of *Gonionemus*. He followed the development of the egg into the hydroid and also the formation of hydroid and medusa-buds from the hydroid. The hydroid does not transform, as Perkins ('03) was inclined to believe, into the medusa. The life history is essentially the same as that of *Craspedacusta*. Here is a form which has always been placed with the Trachymedusæ. It is quite evident that it does not belong there. Such studies emphasize the need of life-history work before we can hope to make a permanent classification of the Hydromedusæ.

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ENDAMŒBA CITELLI SP. NOV. FROM THE STRIPED GROUND SQUIRREL *CITELLUS TRIDECEMLINEATUS*, AND THE LIFE-HISTORY OF ITS PARASITE, *SPHÆRITA ENDAMŒBÆ* SP. NOV.

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ENDAMŒBA CITELLI sp. nov.

In the autumn of 1925 the writer made microscopic examinations of the fæcal mass from the intestines of eight ground squirrels belonging to the species *Citellus tridecemlineatus.* These animals were captured alive at various times in the vicinity of Ames, Iowa, brought to the laboratory, and examined at once for parasitic protozoa. In addition to a number of other interesting protozoa, six of the ground squirrels were found to harbor amœbæ in their cœca. In four of the animals the amœbæ were extremely rare, but two showed extremely heavy infections. The movements of the live amœbæ were studied in normal saline solution, and permanent mounts were made by the well-known Schaudinn-iron-hæmatoxylin method.

The amœbæ live within their host in association with myriads of other protozoa. The habitat is limited to the cœcum and the part of the colon immediately adjoining. Localization in the cœcum has been noted by Kessel (1924) in the case of several species of rat and mouse amœbæ. Kessel thinks the PH relationship has something to do with this localization of habitat. Perhaps another factor is the more fluid content of the cœcum which makes a more favorable medium for amœbæ than the colon, where the water content is very much reduced by absorption.

Examination of the substances in the food vacuoles of the amœbæ show that they feed largely upon bacteria and nondescript particles of undigested vegetable matter. The food vacuoles and cytoplasm often contain a curious parasite of the amœba which resists digestion. This will be discussed below. No red blood corpuscles or tissue cells were ever observed within the cytoplasm. These facts make it fairly safe to conclude that the amœba is a commensal and not a true parasite.

The locomotion of the free forms was studied, because the character of pseudopod formation has come to be of so much importance in correct classification (Dobell, 1921, Kofoid, Swezy, and Kessel, 1923). When kept slightly warmed they exhibited great activity with frequent pseudopod formation. The pseudopods were clear and broadly rounded. The endoplasm did not invade them after they were formed, but they were often withdrawn before the endoplasm had completely filled them. They were not, however, thrust out with the explosive suddenness characteristic of *Endamœba histolytica* which the writer has been fortunate enough to observe on several occasions. Typical "fountain streaming" or limax movement was observed in a number of individuals. The nucleus is not prominently visible in the living specimens, but it can be made out after observing the amœba carefully for a while.

Measurements of stained specimens of the free amœbæ show considerable variation. The smallest one measured eleven by ten micra, and the largest twenty-three by twenty-five micra. An average of ten amœbæ measured was fifteen by sixteen micra, which is somewhat smaller than either *Endamœba coli* or *Endamœba histolytica*, but compares favorably with Kessel's (1924) measurements of *Councilmania decumani* from mice and rats. The nuclei of the free forms measure from four by four micra to six and one tenth by five and two tenths micra in size. Ten measurements averaged four and nine tenths by four and eight tenths micra, which is slightly smaller than the figures given for *E. histolytica* and *E. coli*. (Hegner and Taliaferro, 1924.)

The nucleus is definitely of the vesicular type, with a deeply staining karyosome which varies in position from central (Fig. 6) to extremely excentric (Fig. 1). Usually it is less pronouncedly excentric, as in Fig. 2. This karyosome is surrounded by a clear achromatic zone, which in turn is surrounded by a layer of more or less concentrated slightly basophilic substance (Figs. 1–6; 8). Between this layer and the nuclear membrane is an achromatic reticulum on which are suspended fine basophilic granules. The achromatic nuclear membrane is encrusted on the inner surface by a fine beading of chromatin granules, which is intermediate in coarseness between *E. histolytica* and *E. coli.* A few binucleate individuals were found, but no division figures.

Cysts were exceedingly rare, and only four eight-nucleate cysts could be found; but these were well stained and suitable for study. The shape is nearly spherical (Fig. 9); size, fifteen and one half micra in diameter. Their especially characteristic feature was the thickness of the cyst wall, which was in all cases about one micron. This compares with a thickness of less than 0.5 μ in *E. histolytica* and *E. coli.* The nuclei likewise were fundamentally different from those of the other *Endamœbæ.* All the chromatin from the karyosome and periphery of the nucleus appeared to have collected into a number of irregular, deep-staining blobs. Some of these lay upon the nuclear membrane, while others were farther in the interior of the nucleus. The nuclei of the cyst measured about 2.8 micra in diameter. In contrast to the free forms, where it is coarsely alveolar, the cytoplasm of the cyst appeared granular. Chromatoid bodies were absent, except for a few small dark staining splinter-like bodies in the center of the cyst. The developmental stages of the cyst were not found.

It was upon the basis of thickness of the wall of the cysts, its yellowish tinge, and the character of the cyst nuclei that a new species was created for this amœba. Were it not for this characteristic cyst, it would be difficult to distinguish this from many other *Endamœbæ; e.g., E. muris* (Grassi, 1882) of the mouse and rat.

The writer in a previous paper (1922) pointed out that simply finding a parasite in a host where it had not previously been found was no valid reason for considering it a new species. Especially is this true in the case of amœbæ; for if, as Kessel (1923) determined in his experiments, rodents can be infected with the human amœbæ, it is probable that man can be infected with the amœbæ of rodents. Two points are so sufficiently clear that they should not be ignored in future work in amœbæ. First, is the species being considered sufficiently different morphologically from closely related species so that it should be considered a new one? Second, there is the possibility of man, or domestic animals, becoming the host of an endamœbæ normally found in lower animals. Kessel's work on specificity has encountered severe censure, especially from European workers (*e.g.*, Wenyon). It seems to the writer that the more logical method to criticize the work would be to repeat it, and thus determine if it is really open to such serious defects as has been charged.

SPHÆRITA ENDAMŒBÆ sp. nov.

The name *Sphærita* was given by Dangeard (1886) to a genus of the family Chytridiaceæ, which he considers to represent a transition from animal toward plant forms. Likewise, Doflein (1916) assigns these forms to the borderline between the plant and animal kingdoms, with the additional comment that they must be reinvestigated by one who would study the relationships of the sporozoa, flagellates, and rhizopods. They are of interest to protozoölogists not only because of their phylogenetic relationships to the protozoa, but also because *Sphærita*, and other cythridines, such as *Nucleophaga* (Dangeard, 1895), are parasites upon protozoa, *Sphærita* in the cytoplasm, and *Nucleophaga* in the nucleus. A number of the earlier observers, particularly Stein, Carter, Kent, and de Lanessan misinterpreted the developmental phases of a *Sphærita* within *Euglena* as the production of embryos from the nucleus of the *Euglena*, which grew flagella and later developed into the adult flagellate (see Dangeard, 1886). Dangeard (1886, 1894, 1895) clearly showed that what these authors considered to be the growing and multiplying nuclei of *Euglena* were in reality cythridine parasites of the flagellate for which he established the genus *Sphærita*.

There are not many references to *Sphærita* in the literature. Dangeard proposed the name *Sphærita endogena* for the form found in flagellates (*Euglena*, etc.) and rhizopods (*Nuclearia* and *Heterophrys*). Later Chatton and Brodsky (1909) proposed a separation of the species found in these two groups of protozoa, suggesting that *Sphærita endogena* be retained for the form found in rhizopods, and that the *Euglena* parasite be called *Sphærita dangeardi*. Chatton and Brodsky (1909) described a

Sphærita from *Amœba limax* Dug., which they found to be different morphologically in the younger developmental stages from *S. dangeardi*. No comparison was made with *S. endogena*. Dobell (1919) mentions a *Sphærita* in the free forms of the parasitic amœba, *Endolimax nana*. Kessel (1924) found a *Sphærita* in *Councilmania muris*, entozoic in mice and rats. There are a number of other papers on *Nucleophaga*, closely related to *Sphærita*, except that it is found in the nucleus. It was originally described from *Amœba verrucosa* by Dangeard (1895), but we will not discuss this genus any further here.

The greater number of *Endamœba citelli* from one ground squirrel were parasitized by a species of *Sphærita*. This material showed so great an abundance of individuals in different stages of development that it has been possible to follow almost the complete life-cycle of this interesting cytozoic organism. The earlier stages of development of the parasite were the first to be seen within the cytoplasm of the amœba (Figs. 2, 3, 4). The first impression was that they represented nuclei in the process of construction from chromidia. Further search revealed the large plasmodia with maturing spores (Fig. 7), which led to the correct identification of the bodies as stages of the life-cycle of a *Sphærita*. It would not be surprising if intracellular parasites of protozoa have led observers astray more often than is generally known; *e.g.*, Leidy in Plate VII. of his "Fresh-water Rhizopods of North America" figures a number of specimens of *Amœba villosa* with "large and coarsely granular nuclei," which "nuclei" were probably typical sporangia of a *Sphærita*, the "coarse uniform granules" being the spores. This interpretation is strengthened by Fig. 15 of the same plate, which Leidy describes as representing "collapse of the contractile vacuole and the bursting of one of the nuclei with the simultaneous escape of the granules or spores of the nucleus and the contents of the contractile vacuole." What he probably observed was the liberation of the spores from a sporangium of *Sphærita*.

The life-cycle of *Sphærita citelli* can perhaps best be described by referring frequently to the figures of the plate. Fig. 2 represents an amœba with two parasites in the earlier stages of development. The lower one has a fine cell membrane, which encloses a centrally located, deeply-staining nucleus. The nucleus shows no nuclear membrane or other differentiation, and its diameter is about two thirds that of the cell. The nucleus of the upper specimen has just divided with no apparent spindle or attraction spheres. At this stage the opposing surfaces of the two nuclei are flattened, with the remaining surface of each nucleus convex. The uninucleate and binucleate stages of the cell are about the same size, measuring from 1.9 to 2.5 micra.

A second bipartate division provides the plasmodium with four nuclei with the planes of both divisions still plainly marked (Figs. 3, 4, 18). The organism has become more oval in shape and has increased in size to about 2.5 micra in width and from 2.8 to 3.3 micra in width. From this stage the divisions of the nuclei are not necessarily simultaneous. Specimens were found with eight nuclei (Fig. 5), or with six nuclei, four of them smaller and resulting from the division of two of the nuclei of the four-cell stage, with the other two larger and still undivided (Fig. 19). The plasmodium at this stage measures from 3.7 to 4.0 micra in width to from 4.2 to 5.3 micra in width. Divisions are multiplied until the multi-nucleate spherical stage is attained (Figs. 6, 20, 21). These spheres measure from 5.3 to 8.8 micra in diameter.

The nuclei of the spheres just described stain uniformly black. The next stage in the cycle is the transformation of these nuclei into spores. In this process they enlarge somewhat, stain less intensely, and form a definite spore wall (Figs. 7, 22). Some of them show a thickening of the wall on one side (Fig. 22). These spores usually vary in size from 1.0 to 1.6 micra in diameter. The larger spore in Fig. 22 is exceptionally large, measuring about 1.8 micra. Occasionally spores no larger than 0.5 micron in diameter are found.

A comparison of *Sphærita endamœbæ* with the *Sphærita* from *Amœba limax* so carefully described by Chatton and Brodsky (1909) shows certain fundamental differences. First, the nuclei of the young uninucleate forms are comparatively large and central in *S. endamœbæ*. Those from *Amœba limax* were punctiform and excentric. These distinctions alone are sufficient to justify a distinction between the two species. Second, not all the nuclei of *S. endamœbæ* develop simultaneously into spores, as they apparently do in the form from *A. limax*. Third, there is no nucleus present within the spores of *S. endamœbæ*, although the contents of the spore are clearly visible. Chatton and Brodsky state that it was difficult to see the interior of the spores of their *Sphærita* even in stained specimens. In a few cases, however, they observed an excentric nucleus within the spore. Fourth, the appearances of dividing nuclei of the multinucleate plasmodium dffer in the two species. Those of *S. endamœbæ* are bilobed, or dumb-bell shaped. Those described by Chatton and Brodsky presented the appearance of two cuneiform polar caps. Measurements of the two species in various stages lie within approximately the same limits. The above stated facts make it evident that the *Sphærita* of the *Endamœbæ* is altogether different from that of the free-living amœba, *A. limax*.

Likewise I believe it is different from the one figured by Dobell (1919) in *Endolimax nana*, an amœba entozoic in man. If his figures be correct, the nucleus of the uninucleate stage is punctiform and excentric, as in the parasite of *A. limax*. The morula-shaped mass of spores is likewise not characteristic of *S. endamœbæ*. Kessel's (1924) account is too meagre to afford a comparison.

Chatton and Jansky were not able to determine whether the spores of their *Sphærita* became flagellated, after the manner of the zoöspores of *Sphærita endogena* and *S. dangeardi* as described in the accounts of Dangeard, or remained immobile and were passively ingested by the amœba. Although actual reinfection by the spores was not observed, the writer has been able to follow out the process in his prepared slides.

Among the bacteria present on the slide one occasionally finds dumb-bell shaped organisms, resembling *Azotobacter*. There seem to be two general sizes, one considerably larger than the other (Fig. 10). These multiply by binary fission (Fig. 11). It is not unusual to find these dumb-bell-shaped bacteria-like bodies in the food vacuoles of the amœbæ (Figs. 8, 12, 13). In the food vacuoles the organism undergoes considerable change. Dense granulation appears in the more or less homogeneous cytoplasm. The dark granules collect in a deeply-staining clump in the center of the cell (Figs. 3, 8, 14). The two members of the dumb-bell shaped pair usually separate, and are carried some distance from each other. Finally, the deeply-staining mass of granules becomes compact and uniformly solid (Figs. 2, 3, 8, 15, 16, etc.). By this time the fluid content of the vacuole has disappeared and the wall of the *Sphærita* is contiguous with the cytoplasm of the amœba. The development then proceeds as described above.

The writer realizes that it may be objected that two organisms have been confused in this cycle, and that what has been interpreted as the infective form of the *Sphærita* is in reality a bacterium ingested as food. This danger was a cause of considerable anxiety, and it was not until a large amount of material was studied that the writer was convinced of the specific identity of the two forms. A careful study of such appearances as in Fig. 8 (where one finds a perfect series from the bacterium-like form to the early uninucleate form, unmistakably that of *Sphærita*) brings conviction of the transition from one form to the other. The only gap in the life-history is the failure to observe convincing stages of the growth of the spores into these larger bacterioid forms. It is to be expected that such stages would be exceedingly difficult to find, considering the amount of the fæcal mass in proportion to the number of spores. The writer has observed, however, the smaller dumb-bell-shaped dividing spores resembling the smaller individual in Fig. 10 within an old sporangium from which all but a few of the spores had been expelled.

The extracellular development of *S. endamœbæ* differs in several important respects from that described by Dangeard for the forms which he found in free-living flagellates and rhizopods. Here the zoöspores became elongated and flagellated as they left the sporangium. Then they united in pairs, as in conjugation. The spores studied by the writer were never flagellated when found outside the amœba, and no conjugation of spores was observed, although what appeared to be dividing spores indicating a free multiplication cycle were often found.

As in the case of *Sphærita* living in other protozoa, this *Sphærita* is mildly pathogenic to its host. Most parasitized amœbæ exhibit no degenerative changes of any kind (Figs. 2–6, 8). Some of the more heavily infected ones, however, manifest the ill-effects of parasitism by abnormal nuclear appearances (Fig. 7). The karyosome becomes swollen and irregular in shape.

The chromatin beading on the nuclear membrane collects into thick, elongated, deeply-staining blobs. Dobell (1919) also figures nuclear degeneration in parasitized *Endolimax nana*.

SUMMARY.

1. *Endamœba citelli* sp. nov. is a commensal in the cœcum of the striped ground squirrel, *Citellus tridecemlineatus*.

2. The nucleus of the free form is typical of the genus *Endamœba*.

3. The cyst is characterized by a nuclear structure somewhat different from that known for other amœbæ, and an unusually thick cyst wall with a yellowish refraction.

4. The cytoplasm of this amœba may contain a cytridine parasite, *Sphærita endamœbæ* sp. nov.

5. The developmental cycle of this cytozoic parasite was followed from the free bacterium-like infective stage to the spore liberated from the sporangium inside the cytoplasm of the amœba.

6. *Sphærita endamœbæ*, like other members of the genus, produces degenerative changes in the protoplasm of its host, particularly in the nucleus.

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EXPLANATION OF PLATE.

× 1420.

FIG. 1. Free form of *Endamœba citelli* with typical nucleus fixed with two pseudopods extended. Two food vacuoles in cytoplasm contain respectively a bacterium and a partially digested yeast.

FIG. 2. Amœba infected with young uninucleate and binucleate *Sphærita*.

FIG. 3. Amœba with same stages plus one younger individual with nucleus in process of formation and an individual with four nuclei.

FIG. 4. Large amœba with one parasite having four nuclei.

FIG. 5. *Sphærita* has eight nuclei, each dumb-bell-shaped preparatory to division.

FIG. 6. Multinucleate *Sphærita* in cytoplasm of amœba.

FIG. 7. Large sporangium with nuclei developing into spores.

FIG. 8. Amœba with large food vacuole containing infective bacterium-like stages developing into typical uninucleate cytozoic forms.

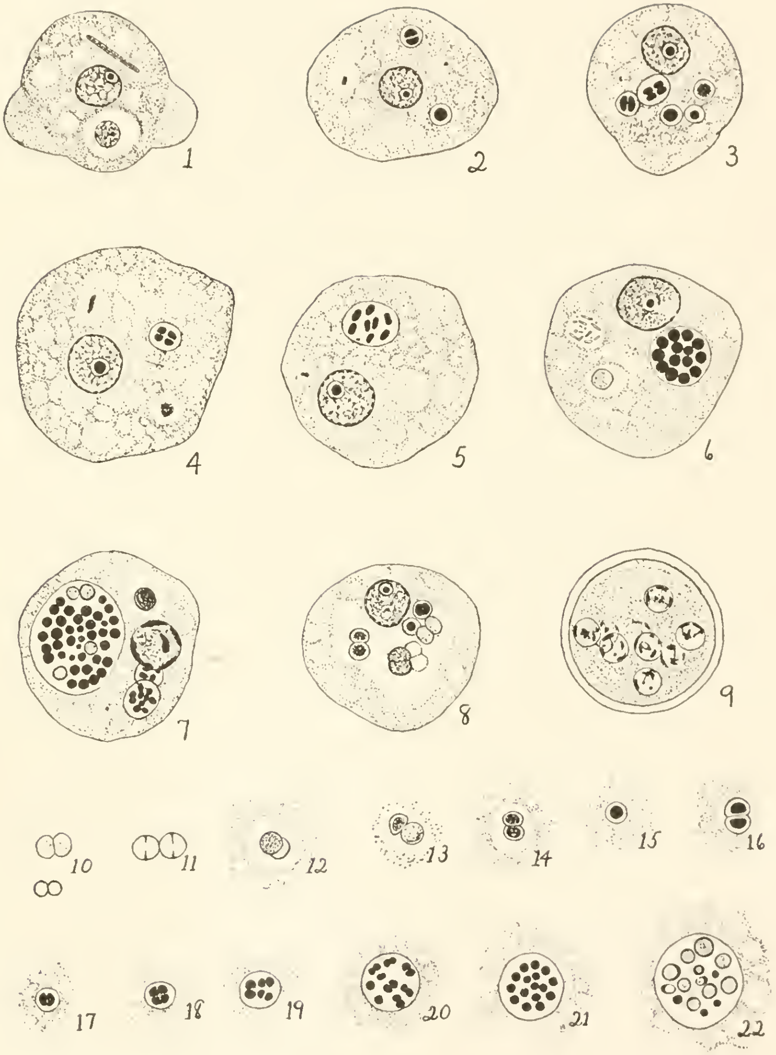
FIG. 9. The eight-nucleate cyst of *E. citelli.*

FIGS. 10–11. Free bacterium-like stages of *Sphærita endamœbæ.*

FIGS. 12–14. Bacterium-like stages in food vacuoles of the amœba.

FIGS. 15–22. Developmental cycle of *Sphærita* in cytoplasm of amœba. Fig. 16 does not represent a division of the organism in Fig. 15, but rather the same stage of development, the next stage after Fig. 14. The two members of the pair later separate.

PLATE I.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ELERY R. BECKER.

MODIFICATION OF DEVELOPMENT ON THE BASIS OF DIFFERENTIAL SUSCEPTIBILITY TO RADIATION.

III. *Arbacia* Germ Cells, and (*a*) Ultraviolet Radiation, (*b*) Visible Radiation Following Sensitization.

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Experiments made with x-rays, radium, ultraviolet radiation, and visible light (following sensitization),[1] clearly demonstrate that radiation may produce an injurious effect on living tissues provided certain conditions are satisfied, namely; (*a*) the wave-length range of radiation impinging on the tissue must include that in which the tissue absorbs; (*b*) the spectral energy content must be of sufficient magnitude, and (*c*) the duration of the exposure must be long enough, to insure efficiency of action.

When intensity and duration of exposure to a given radiation, *e.g.*, ultraviolet radiation, are so regulated as to produce almost immediate death, it is found that there is an antero-posterior gradient of death and disintegration coincident (in lower animals and early embryonic stages) with the main body axis. Those regions of the body which are physiologically the most active are the first to die and disintegrate. In other words, there is a differential susceptibility to radiation in living organisms. (See also Bovie and Barr, '24, Hinrichs, '24, and Child and Deviney, '26.)

On the other hand, a sublethal dose of radiation applied in early embryonic stages, will differentially modify the later development. Here again, the regions of high physiological activity are first to be modified, and may be permanently inhibited, or if the injurious action be only slight or transitory, these regions may show recovery or acclimation more rapidly than other regions. By regulating the dosage, differential inhibition or differential acclimation (or recovery) may be obtained.

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[1] See Clark, '22, Colwell and Russ, '24, Dubois, '14, Ellis and Wells, '25, Hausmann, '23, Hinrichs, '25, '26, L. Loeb, '22, for review of literature.

Such experiments have been made with (*a*) *Fundulus* and ultraviolet radiation, Hinrichs, '25, (*b*) *Arbacia* and ultraviolet radiation, Child, '24, footnote, p. 109, Hinrichs, '26, and (*c*) *Arbacia* and visible radiation following sensitization with eosin, neutral red, benzoflavine, and methylene blue, Hinrichs, '26. So far, experiments with radiation have given results entirely parallel with those produced by chemical and other physical agents, thereby further confirming the view of the nonspecific and quantitative nature of susceptibility relations along the body axis.

In previous experiments it was found that the larval development of *Arbacia* may be modified by subjecting the fertilized egg, preferably during the first few minutes after insemination, to doses of radiation which, although not immediately lethal, may produce sufficient injury to interfere with the normal succession of developmental processes. In further experiments with *Arbacia*, the same types of differential modification were produced by subjecting either component of the zygote, before fertilization, to the proper dosage of radiation.

Much experimental work has been reported in which chemical treatment of either or both sex cells before insemination has brought about abnormal development. Stockard, in a series of papers, reports the production of abnormalities through the mating of alcoholized guinea pigs. (See Stockard and Papanicolaou, '18, for references to previous papers.) Rondeau and Luzeau, '01, with isotonic NaCl and sugar solutions on the eggs of *Rana fusca*, Gee, '16, with alcohol and NaOH on the germ cells of *Fundulus*, and Dungay, '13, with distilled water, chemicals, heat and cold, on *Nereis* and *Arbacia* sperm, were in each case able to produce modification of larval development; and the latter author recorded distinct differential effects. Bohn and Drzewina, '23, were able to diminish motility in the sperm of *Strongylocentrotus* by the combined action of neutral red and light, and also by the use of KCN, KCl, and of distilled water (Drzewina and Bohn, '12, '23, *a*, *b*). O. Hertwig, '13, using chloral hydrate and strychnine nitrate on the sperm of *Rana fusca*, was able to produce spina bifida, and a general delay of organ-formation and hatching. He points out the similarity of the effects resulting from the separate treatment of either eggs or sperm. (See also G. and P. Hertwig, '13.) The latter authors, by exposing the sperm of frogs, fish, and sea urchins, to the action of chloral hydrate, strychnine nitrate, and methylene blue, were able to obtain delayed cleavage and modified development. They report, as a result of fertilizing the eggs of *Rana esculenta* by sperm of *Rana fusca*, previously treated with chloral hydrate, the appearance of short, œdematous larvæ with bent tails (and some with spina bifida) and poorly developed eyes. They further state that sperm may be injured without impairment of motility and that the sperm chromatin, although injured, remains in the egg during cleavage. This fact is further substantiated in their work on *Strongylocentrotus lividus* and *Sphærechinus granularis;* they fail to find evidence of the induced parthenogenesis which others have described.

A number of experimenters have exposed one or the other sex component to the action of radium or x-rays. Bardeen, '07, '09, modified Amphibian development by exposing sperm and eggs to radium and x-rays. His results indicate a modification of development of those body regions where growth and complex differentiation are normally most rapid, *e.g.*, neural tube, eyes, nose, and heart, *i.e.*, differential inhibition. Oppermann, '13, studied the effects of radium radiation on sperm as well as on fertilized eggs of trout. He records defects of the eyes and head and abnormal development of the tail region. G. Hertwig, '11, '12, '13, found that prolonged exposure of the sperm of frogs and *Arbacia* to radiation injures the chromatin so that it cannot take part in cell division. When unfertilized frogs' eggs are subjected to radium treatment and are then fertilized by normal sperm, the developing larvæ show the greatest modification in the developing sense organs, muscle plates, and blood cells. O. Hertwig, '11, '13, using radium on the germ cells of the frog, and the eggs of *Triton* was able to obtain differentially modified forms. P. Hertwig, '11, found that the chromatin in radium-treated *Ascaris* eggs was affected before the cytoplasm, and that cleavage was delayed and irregular and death appeared early. She also studied the behavior of the chromatin of radium-treated frog sperm, '13, and found that the sperm nucleus took no part in the formation of the zygote nucleus nor in subsequent cleavage figures. Packard, '18, found that radiation of the egg of *Chætopterus* before fertilization prevents the egg nucleus from taking part in division, although the sperm nucleus behaves normally. Richards and Good, '19, found that radiation of *Cumingia* sperm did not affect the rate of cleavage, and that radiation of fertilized eggs produced first an acceleration in the rate of cleavage followed by a retardation. On the other hand, brief radiation of the unfertilized egg produced retarded cleavage and abnormal development. Exposure of sperm was less effective than exposure of unfertilized eggs. Redfield and Bright, '21, have shown that exposure of *Nereis* eggs to ultraviolet radiation interferes with the production of normal membranes.

In some of the above-cited work where the later development was observed, it is interesting to note that the departure from normal development appeared in systems and organs which are known to have a high rate of physiological activity. Similar conditions are found when either or both of the germ cells of *Arbacia* are exposed to ultraviolet radiation, or to visible radiation following sensitization. The region of the egg which is destined to give rise to the oral lobe and the anterior end of the pluteus has its normal development modified to a greater degree than the other parts of the egg. The resulting embryos show greater inhibition or even absence of development of the oral lobe and aboral arms.

Method.—As in previous work, two regions of the spectrum were used, the ultraviolet region, and the visible region following sensitization.

Ultraviolet Radiation was obtained from a quartz Hg-vapor arc (Cooper Hewitt), running at 60 volts, 4.0 amps. The experimental material, in open dishes, was placed approximately 30 cm. below the center of the arc. The above source is rich in ultraviolet radiation, although its spectrum extends well into the visible region (1,850–7,700 Å.).

Visible Radiation.—In this group of experiments, eggs or sperm were exposed in weak concentrations of dyes (1/2,000–1/20,000), to radiation from one of the following light sources;

1. A 1,500-watt Tungsten filament lamp burning tip-down at a

distance of 25–50 cm. above the experimental material. In some experiments, a second lamp burning tip-up was simultaneously used at the same distance below the experimental material. Temperature was controlled by the addition of ice to the water-bath surrounding the dishes. A glass dish containing a one-inch layer of water filtered out the injurious ultraviolet and heat rays.

2. A 100-watt condensed-filament lamp (of the type used with the Bausch and Lomb euscope) running at 6 volts, was placed at a distance of 25 cm. from the substage mirror of a microscope, with a water filter interposed between the lamp and the mirror. The dish containing the experimental material was placed on the microscope stage and samples of eggs or sperm were withdrawn at intervals from the center of the region reached by the light reflected up through the condenser and dish.

3. In some experiments a carbon arc, water filter, and glass lens were used in place of the above arrangement. Samples were withdrawn from the lighted portion at the focal point of the lens.

4. Direct sunlight (on mid-August afternoons) filtered through a one-inch layer of water in a glass dish was used in another set of experiments. A water bath surrounding the experimental dishes further controlled the temperature.

5. Diffuse daylight near a north window was used in another series. The dishes were covered with glass plates.

In all experiments, eggs or sperm were exposed in a thin layer just covering the bottom of the dish (about 2 mm. deep). The maximum effective periods of exposure and the intervals between the removal of successive samples were necessarily shorter with the intense radiation of the artificial light sources, than with direct sunlight or diffuse daylight. Typically, eggs fertilized by sperm which had been exposed for 45 minutes to direct sunlight showed merely a differential acclimation following a slight inhibition; while when sperm was used which had been exposed for only 5 minutes (under the above conditions) to radiation from Tungsten bulbs or the carbon arc, the result was differential inhibition with no recovery in a large proportion of the embryos.

In the experiments with visible radiation, stained and unstained sperm (or eggs) were radiated, and a parallel unradiated control series was kept in the dark. There were further controls as follows; (*a*) in the light (in diffuse daylight throughout the experiment), and (*b*) in the dark, of each of the following: normal eggs fertilized by normal sperm (stained and unstained), unfertilized eggs (stained and unstained). This gave a check on accidental fertilization during preparation of the material, also on any possible injurious effect of the dyes. The norm for the series was furnished by the embryos resulting from the fertilization of normal eggs by normal sperm.

The results obtained with visible radiation following sensitization, and with ultraviolet radiation were essentially similar, except that the length of the effective periods of exposure to ultraviolet radiation was always much shorter; for example, differential inhibition without recovery could be obtained by exposing sperm (1/3,600 per cent.) for 10–15 seconds.

Results.—Since exposure of either sex component (before fertilization) to radiation from any one of the various light sources produces differential modification of development, and since the degree of modification is in general proportional to the dosage of radiation, it seems best to consider the results obtained all together, and to describe first the types obtained, and later the conditions under which one type or the other predominated.

The relative effectiveness of the various light sources may be determined by noting the duration of exposure required in each case to produce a given proportion of differentially inhibited forms.

A. Differential Inhibition.—Long-continued or very strong radiation of eggs (fertilized or unfertilized), and of sperm before using in the fertilization of normal eggs, produces embryos which show marked evidences of permanent differential inhibition. They are characterized by having the development of the oral lobe, aboral arms, and the region between them, more or less impaired or even entirely suppressed (Figs. 3–24).[1] Inhibition of development of the median anterior region results in the production of plutei in which the aboral arms are closer together than normal (Fig. 4), closely parallel (Figs. 5–7), or even partially or completely fused in the median line (Figs. 8–16). In these experiments, such forms have been obtained by exposing sperm in relatively great dilutions (1/300 per cent. to 1/6,000 per cent.), as well as with more concentrated sperm ($\frac{1}{3}$ per cent.), such radiated sperm being then used to fertilize normal eggs. The proportion of differentially inhibited forms for a given dosage is greater when the more dilute sperm is used. The same type of result, *i.e.*, differential inhibition, appears when eggs are radiated before fertilization with normal sperm. In such cases, however, under similar conditions of radiation, the percentage of permanently inhibited forms and delayed cleavage is considerably less than in the case of radiated sperm. Radiation of either sex component before fertilization, delays and often interferes with normal cleavage so that gastrulation is physically impossible. (See Table I.)

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[1] Figures were drawn with the aid of a Bausch and Lomb euscope, and represent an approximate magnification of 48 diameters, following reduction.

TABLE I.

EFFECT OF RADIATION ON CLEAVAGE.

A. Sperm—Visible Radiation.					*B*. Eggs and Sperm—Ultraviolet.			
Time Exposed in Min.	Eosin.	Benzo.	N. Red.	M. Blue.	Time Exposed in Sec.	1/3,600% Sperm.	Time Exposed in Sec.	Eggs.
0	100[1]	100	100	100	0	100	0	100
3	20	100	8	75	2	50	5	100
5	8	100	0	50	6	10	15	98
10	0	90	0	5	10	1	45	75

In many cases exogastrulæ are formed (Figs. 17–19). Frequently inhibition is carried to the point where the characteristics of normal plutei are completely obliterated, and the resulting larva appears as a spherical, apolar mass with non-directive swimming, with or without short skeletal arms imbedded in the tissue (Figs. 20–24).

When sperm are exposed for one and a half minutes to ultraviolet radiation, in the case of dilute sperm, or for two and a half minutes, in the case of concentrated sperm, the eggs show 80 to 100 per cent. of delayed cleavage, followed by the production of differentially inhibited larvæ. (See Table II.)

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[1] Figures indicate the percentages of developing eggs which have reached the early blastula stage. The light source was a 1,500-watt bulb, at a distance of 25 cm. Sensitizing dyes were used in 1/2,000 concentration.

TABLE II.

Effect of Ultraviolet Radiation of Sperm on Development.

	A. Dilute Sperm.				*B*. Concentrated Sperm.			
Time Exposed in Sec.	N.	Accl.	Inh.	D.	N.	Accl.	Inh.	D.
30	90[1]	5	5	0	80	15	5	0
60	60	20	20	0	60	25	15	0
90	40	20	40	0	40	30	30	0
120	0	30	50	20	10	20	70	0
150	0	20	20	60	5	10	80	5
180	0	2	8	90	2	10	78	10

When eggs are exposed to ultraviolet radiation for 1 to 4 minutes, and are then fertilized by normal sperm, typically 60–80 per cent. of the resulting larvæ are normal, 10–20 per cent. show indications of recovery, about 10 per cent. are distinctly inhibited, and about 10 per cent. are dead. When the exposures exceed 5 minutes, the mortality rises to about 70 per cent., with an even distribution of normal, inhibited, and recovered forms. Long exposures interfere so seriously with cleavage that death results in early stages. The controls may be in the blastula stage, while the radiated eggs show irregular 2- and 4-cell stages.

B. Differential Acclimation and Differential Recovery.—Forms showing differential acclimation, where exposures of sperm have been slight enough to permit adjustment of the system (*i.e.*, of normal egg × radiated sperm) to new developmental conditions, and forms showing differential recovery, where inhibition due to radiation of either egg or sperm is merely transitory, show the opposite type of development from those which are permanently differentially inhibited. There may be merely an overdeveloped oral lobe, with aboral arms normal (Figs. 25–26), or the latter may appear smaller than normal (Fig. 27), or there may also be evidence of regulation in the median anterior region, as indicated by the spreading of the aboral arms (Figs. 27–28). Sometimes the angle between the arms reaches 180° (Figs. 29–30).

It is possible to obtain a good percentage of differential acclimation by fertilizing normal eggs with sperm which have had either a short exposure to high intensities, or a longer exposure to low intensities of radiation,—as for example, with sperm exposed for 45 to 60 minutes in diffuse daylight. Differential recovery may be produced by radiating eggs intensely for a short period, or less intensely for a longer period (Figs. 31–38).

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[1] Figures indicate the percentages of developing plutei which are normal (N.) acclimated (Accl.), inhibited (Inh.), or dead (D.).

Eggs stained with either of the four dyes (1/5,000) and exposed at a distance of 50 cm. for 15 minutes to radiation from a 1,500-watt lamp typically showed evidence of slight recovery in a large proportion of cases. As a rule, with exposures of 30 minutes or longer, only about 20 per cent. of the eggs remained alive, and of these a certain proportion (presumably the shaded minority) formed normal plutei. Only a small proportion of the plutei showed evidence of differential recovery after an exposure of 45 minutes. When unfertilized eggs are radiated as above, and are then fertilized by normal sperm, the proportion of abnormal larvæ which develop is smaller than in the case of eggs which are fertilized before they are radiated.

In this series, the photodynamic action of benzoflavine and eosin was less pronounced than that of neutral red and methylene blue.

Differential Inhibition Followed by Differential Acclimation or Recovery.—In experiments with radiation, particularly with ultraviolet radiation, where exposures are exceedingly short (a few seconds to 2 minutes), it is possible to have a primary inhibition followed by a secondary acclimation (Figs. 39–45) or recovery (Figs. 50–52). The oral lobe region recovers more rapidly than do other regions. In any case, the form of the larva indicates that there has been a relatively more rapid return or approach to the normal rate of physiological activity and growth of these highly susceptible regions as compared with less susceptible ones. Consequently the gradient is steepened, and the more apical regions, particularly the oral lobe, are over-developed in relation to the more basal regions, which are under-developed.

Occasionally forms appear in which more than the normal number of aboral arms are present (Figs. 46–49). Such an over-development of the skeleton is probably a result of differential inhibition persisting after general recovery. (See Child, '16, p. 115, and Hinrichs, '26.)

Discussion.—The differential modification of development produced in *Arbacia* eggs by radiation resembles so closely that produced by chemicals and other agents (Child, '16, '24, MacArthur, '24, Hinrichs, '26), that a lengthy discussion appears unnecessary here. Since the effect is differential, we may infer that a difference in susceptibility relations is established at an early stage along the axis of the egg. The results are the same whether the egg is fertilized or unfertilized at the time of radiation. Those regions of the egg which have the highest rates of physiological activity and are consequently the most intimately dependent on the continuance of normal conditions, or as R. S. Lillie puts it ('23, p. 39), on a proper "coöperation of external and internal factors," are naturally the first to show failure of equilibrium or incompatibility in their relation to their environment. (See also L. Loeb, '22.) The result is an interference, temporary or permanent, with the normal development of these regions, the degree of interference for a given dosage of radiation being in general proportional to the intimacy of dependence of the region on its environment. Such regions of high activity are also the first to acclimate or recover, provided the degree of inhibition has not been such as to produce permanent injury.

In the cases where normal eggs are fertilized by radiated sperm, the injurious action of the radiation on the sperm prevents normal development of the egg. Whether this is due to the bringing in of the toxic sperm protoplasm alone, which may be conceived of as acting in the same manner as other agents which interfere with normal development, or whether the abnormal development is an expression of incomplete fertilization bordering on parthenogenesis, as suggested by P. Hertwig, '13, is an open question which these experiments have raised but not answered. (In this connection, see also Lillie and Baskervill, '22*a*, F. R. Lillie, '11, '12, Oppermann, '13, and Dungay, '13.) I am inclined to believe that the toxic action of the radiated sperm protoplasm (after its incorporation with the egg protoplasm to form the zygote), induces abnormal development of the zygote in the same manner as do other toxic agents. There seems to be a certain analogy between this type of abnormal development and that resulting from hybridization (Newman, '17).

These experiments with sperm add further evidence for the non-specificity of susceptibility relations along the egg axis, since the characteristic difference in susceptibility is shown when the radiation acts directly on the egg protoplasm as well as when radiated sperm is used in place of normal sperm to fertilize the normal egg. On the one hand, there is a differential susceptibility to radiation, and on the other, to the action of radiated sperm. The response on the part of the egg protoplasm is the same in both cases, a differential inhibition, recovery, or acclimation. Although we find a difference in the degree of susceptibility to a given dosage of radiation, when we compare the fertilized egg, the unfertilized egg, and the sperm, in general we find that the resting unfertilized egg is less susceptible to a given dosage than the sperm before union with the egg, or than the egg when exposed immediately after union with the sperm.

The action of radiation on the sperm is shown by the reduction and frequent loss of motility (see also Lillie and Baskervill, '22*a*), as well as by the interference with normal cleavage and development. When stronger doses of radiation are used, the fertilizing power is also reduced. (See later paper for fuller discussion.)

The differential effects produced by fertilizing normal eggs with stained sperm previously exposed to sunlight or to diffuse daylight are probably referable to a summation effect of "staling" superposed on injury by radiation. In such experiments the exposures often exceeded 30 minutes or even an hour, so that although the sperm was highly concentrated when exposed, there was probably a slight falling off in normal fertilizing power during that period.[1] (See F. R. Lillie, '15, for study of effect of time and dilution on fertilizing power of sperm.)

These experiments were made at Woods Hole during the summers of 1924 and 1925, and I wish to thank Dr. R. S. Lillie, under whose direction the work was done, for his interest and coöperation.

Conclusions.—From these results, further evidence is obtained for the following:

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[1] G. and P. Hertwig, '13 report two-hour exposures of the sperm of *Sphærechinus* and *Strongylocentrotus* to the action of chloral hydrate, methylene blue, etc. A two-hour delay in the use of *Arbacia* sperm results in considerable loss of fertilizing power, even under normal conditions.

1. In order that visible radiation following sensitization may be effective, the sensitized system, in this case eggs or sperm before fertilization, must be exposed to radiation of sufficient intensity and duration whose wave-length range includes that absorbed by the particular sensitizer used.

2. Ultraviolet radiation is effective without the aid of a sensitizer, by virtue of its direct absorption by protoplasm.

3. Susceptibility to sublethal doses of radiation in these two spectral regions is a differential one, *i.e.*, regions of high physiological activity are the first to be modified in their development. They are also the first to recover when the injurious effect is slight.

4. Modification of development by means of radiation produces results essentially similar to those obtained by other means.

5. Development may be modified by subjecting fertilized eggs, soon after insemination, to the action of intense radiation as in previously reported experiments. Modification of development may also be obtained by exposure of either sex component of the zygote to intense radiation previously to fertilization.

6. A differentially modified larva resulting from the union of radiated eggs and normal sperm is evidence that differences in the susceptibility of the various regions already exists in the unfertilized egg.

7. A differentially modified larva developing from a zygote the sperm component of which has been injured by radiation may be either differentially inhibited or differentially acclimated.

8. Since the developing embryo becomes differentially modified, it appears that the egg at the time of fertilization already shows a difference in susceptibility of its various regions to the injurious action of the radiated sperm.

9. Radiation of sperm reduces its motility, delays cleavage, and interferes with normal development of the zygote.

10. Radiation of sperm inhibits its fertilizing power. (See later paper.)

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DESCRIPTION OF FIGURES.

PLATE I. FIGS. 1–30.

Normal Larvæ.—Figs. 1–2. Fig. 1, aboral, and Fig. 2, lateral view of normal pluteus, 48 hours after fertilization.

Differential Inhibition.—Figs. 3–24.

FIG. 3. Eggs exposed 45 minutes in 1/5,000 Eosin. 1,500-watt bulb, at 50 cm.

FIG. 4. Eggs exposed 15 minutes in 1/5,000 N. red. 1,500-watt bulb, at 50 cm.

FIG. 5. Unstained sperm 10 minutes. 1,500-watt bulb, at 25 cm.

FIG. 6. Concentrated sperm 1 minute. Ultraviolet.

FIGS. 7–8. Eggs exposed 45 minutes in 1/5,000 N. red. 1,500-watt bulb, at 50 cm.

FIG. 9. Sperm exposed 10 minutes, natural pigment. 1,500-watt bulb, at 50 cm.

FIGS. 10–11. Sperm (dilute) 2½ minutes. Ultraviolet.

FIG. 12. Sperm (conc.) 2 minutes. Ultraviolet.

FIG. 13. Sperm exposed 30 minutes, natural pigment. 1,500-watt bulb, at 50 cm.

FIG. 14. Sperm exposed 30 minutes in 1/10,000 Eosin. 1,500-watt bulb, at 50 cm.

FIG. 15. Eggs exposed 45 seconds. Ultraviolet.

FIG. 16. Eggs exposed 45 minutes in 1/5,000 N. red. 1,500-watt bulb, at 50 cm.

FIG. 17. Eggs exposed 15 minutes in 1/5,000 Benzo. 1,500-watt bulb, at 50 cm.

FIGS. 18–19. Sperm exposed 30 minutes in 1/10,000 Eosin. 1,500-watt bulb, at 50 cm.

FIG. 20. Sperm (conc.) 3 minutes. Ultraviolet.

FIG. 21. Sperm exposed 2 seconds. Ultraviolet.

FIG. 22. Sperm exposed 30 minutes in 1/10,000 Eosin. 1,500-watt bulb, at 50 cm.

FIG. 23. Sperm (conc.) 2 minutes. Ultraviolet.

FIG. 24. Sperm (conc.) 2½ minutes. Ultraviolet.

Differential Acclimation.—Figs. 25–30.

FIG. 25. Sperm (dil.) 1 minute. Ultraviolet.

FIG. 26. Sperm (conc.) ½ minute. Ultraviolet.

FIG. 27. Sperm 10 minutes, natural pigment. 1,500-watt bulb, at 50 cm.

FIG. 28. Sperm (conc.) 1 minute. Ultraviolet.

FIG. 29. Sperm 10 minutes, unstained. 1,500-watt bulb, at 25 cm.

FIG. 30. Sperm 10 minutes in 1/10,000 Eosin. 1,500-watt bulb, at 25 cm.

PLATE I.

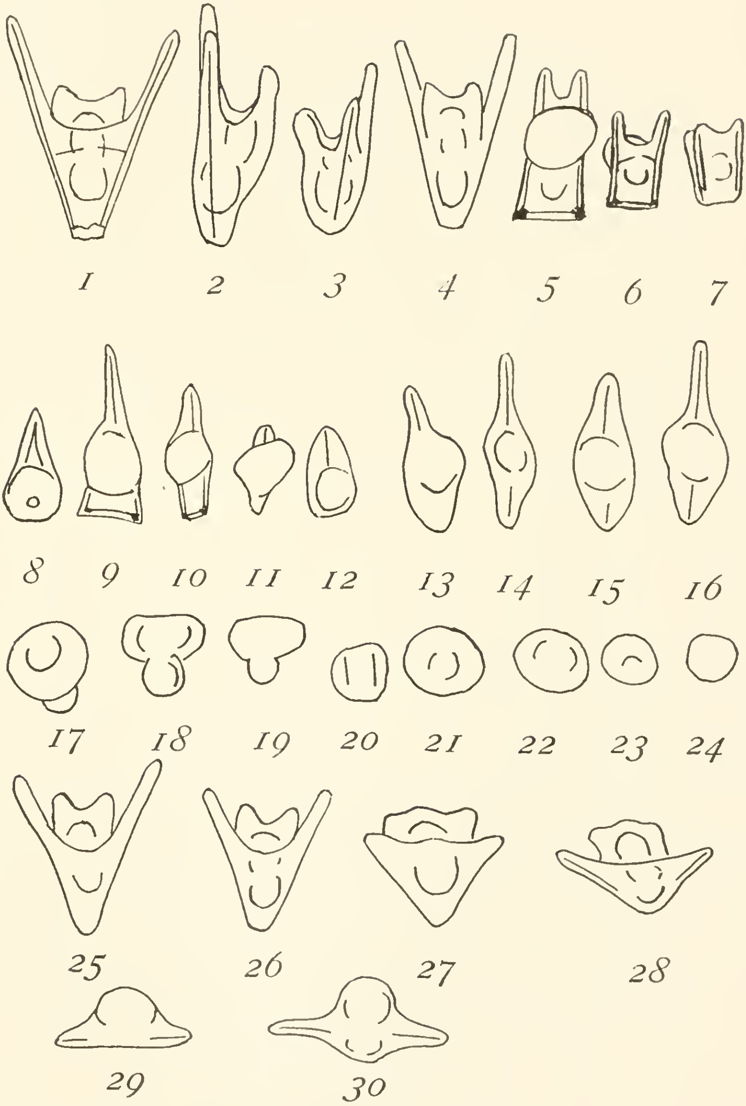
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PLATE II. FIGS. 31–52.

Differential Recovery.—Figs. 31–38.

FIG. 31. Eggs exposed 15 minutes in 1/5,000 Benzo. 1,500-watt bulb, at 50 cm.

FIG. 32. Eggs exposed 45 minutes in 1/5,000 M. blue. 1,500-watt bulb, at 50 cm.

FIG. 33. Eggs exposed 15 minutes in 1/5,000 Benzo. 1,500-watt bulb, at 50 cm.

FIG. 34. Eggs exposed 45 minutes in 1/5,000 M. blue. 1,500-watt bulb, at 50 cm.

FIG. 35. Eggs exposed 15 minutes in 1/5,000 Benzo. 1,500-watt bulb, at 50 cm.

FIG. 36. Eggs exposed 45 minutes in 1/5,000 Benzo. 1,500-watt bulb, at 50 cm.

FIG. 37. Eggs exposed 45 minutes in 1/5,000 N. red. 1,500-watt bulb, at 50 cm.

FIG. 38. Eggs exposed 15 minutes in 1/5,000 N. red. 1,500-watt bulb, at 50 cm.

Differential Inhibition Followed by Acclimation.—Figs. 39–49.

FIG. 39. Sperm (conc.) ½ minute. Ultraviolet.

FIG. 40. Sperm (dil.) 3 minutes. Ultraviolet.

FIG. 41. Sperm (conc.) 3 minutes. Ultraviolet.

FIG. 42. Sperm 10 minutes in 1/10,000 Eosin. 1,500-watt bulb, at 50 cm.

FIG. 43. Sperm 30 minutes unstained. 1,500-watt bulb, at 25 cm.

FIG. 44. Sperm (conc.) ½ minute. Ultraviolet.

FIG. 45. Sperm (dil.) 2½ minutes. Ultraviolet.

FIG. 46. Sperm (conc.) 1 minute. Ultraviolet.

FIG. 47. Sperm 10 minutes in 1/10,000 Eosin. 1,500-watt bulb, at 50 cm.

FIG. 48. Sperm 30 minutes in 1/10,000 Eosin. 1,500-watt bulb, at 50 cm.

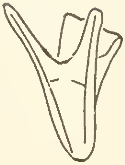
FIG. 49. Sperm 30 minutes unstained. 1,500-watt bulb, at 25 cm.

Differential Inhibition Followed by Recovery.—Figs. 50–52.

FIG. 50. Eggs exposed 15 minutes in 1/5,000 Benzo. 1,500-watt bulb, at 50 cm.

FIG. 51. Eggs exposed 45 minutes in 1/5,000 Benzo. 1,500-watt bulb, at 50 cm.

FIG. 52. Eggs exposed 45 minutes in 1/5,000 Benzo. 1,500-watt bulb, at 50 cm.



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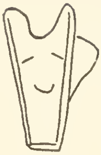

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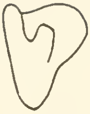

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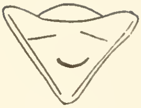

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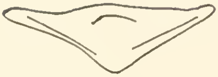

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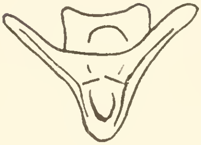

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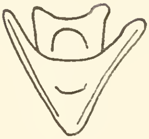

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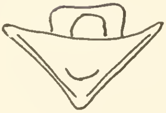

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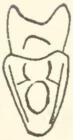

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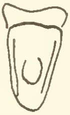

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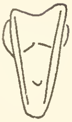

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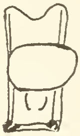

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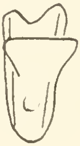

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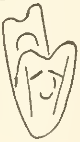

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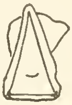

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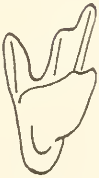

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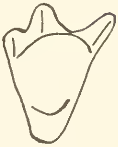

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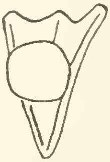

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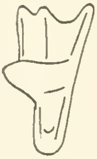

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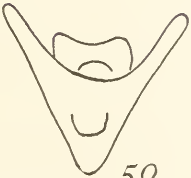

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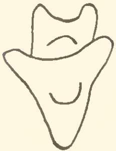

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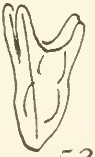

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MARIE A. HINRICHS.

THE EFFECT OF ULTRAVIOLET RADIATION ON THE FERTILIZING POWER OF *ARBACIA* SPERM.

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It is a well established fact that ultraviolet radiation may have an injurious effect on living tissues under certain conditions. That *Arbacia* sperm is no exception is evident from the diminution of fertilizing power following exposure of sperm to radiation from a quartz mercury-vapor arc whose spectrum range covers approximately 1,850–7,700 Å.

A number of chemical and physical agents have been used to injure the sperm of various animals. In general, such treatment results in a decrease of motility and a lowering of fertilizing power. In eggs which have been fertilized by treated sperm, cleavage is delayed and irregular, gastrulation often impossible, and development abnormal, particularly in the regions of relatively high physiological activity.[1]

Sperm may be considered as being endowed with a definite and limited amount of available energy which is expended at a ratio determined by its activity. Cohn, '18, found that the total amount of carbon dioxide produced during the lifetime of sperm was constant; the more active the sperm, the shorter its life. The same investigator (Cohn, '17) found that sperm in acidified water retained their fertilizing power for a long time, although they remained relatively inactive.

Certain chemical agents are known to stimulate sperm to greater activity, *e.g.*, alkalies, thereby decreasing the length of life of the sperm. (See Gray, '15, and Gee, '16.) Certain investigators have found that eggs give off substances which stimulate sperm to greater activity. Fuchs, '14, found that the blood of freshly caught animals increased the fertilizing power of certain sperm. The same author, in a study of cross-fertilization

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[1] For a review of the literature in this connection, see previous paper, Hinrichs, '26*b*.

(Fuchs, '15), found that some egg secretions may produce an increase in the fertilizing power of the sperm of another species. Clowes and Bachman, '20, have obtained a volatile sperm-stimulating substance from *Arbacia* eggs.

On the other hand, a number of agents have been used to inhibit the activity of sperm, for example, neutral red in the presence of light, ultraviolet radiation, radium, temperature, x-rays, the electric current, and various chemicals.[1] (See Bohn and Drzewina, '23*b*, Lillie and Baskervill, '22, Oppermann, '13, Dungay, '13, Bardeen, '07, Günther, '07, Cohn, '17, Gee, '16, and others for references.) KCN, which interferes with normal oxidation processes, was found by Drzewina and Bohn, '12, to inhibit the activity of sea urchin sperm as well as to cause irregular cleavage and abnormal development of eggs fertilized by the treated sperm. The ageing of sea urchin germ cells has been found by Goldfarb, '17, '18, to proceed more rapidly after their removal from the body. Correlatively motility and fertilizing power are reduced. Hyperalkaline sea water accelerates the ageing process.

Time and dilution are both known to be factors affecting the fertilizing power of *Arbacia* sperm (F. R. Lillie, '15*b*). Drzewina and Bohn, '23*b*, also showed dilution to be a factor in the susceptibility of sea urchin sperm to the combined action of neutral red and light. Motility and fertilizing power were lost more quickly in dilute than in concentrated sperm suspensions. Usually loss of motility is associated with loss of fertilizing power, but the two do not exactly parallel each other. Fertilizing power is not a function of motility alone, and declines more rapidly than does motility. (See F. R. Lillie, '15*b*, and Lillie and Just, '24.) Sperm may be injured in such a way that its fertilizing power, as measured by the proportion of eggs fertilized and the normality of the cleavage and development, is materially lessened while motility is not visibly impaired. (See also Hinrichs, '26*b*, and Lillie and Baskervill, '22.)

[1] It has been repeatedly pointed out, especially by O., G., and P. Hertwig, (see previous paper for references) that the effect of injury on the sperm is particularly felt by the nuclear material. Packard, '14, however, suggests also a possible effect on the cytoplasm. He postulates the presence of enzymes which may be injured by radiation.

Method.—In these experiments, measured dilutions of sperm (1 per cent. to 1/240 per cent.) were exposed, as a thin film just covering the bottom of the dish, to radiation from a Cooper Hewitt quartz mercury-vapor arc at a distance of 30 cm. for one minute. (Temperature was controlled by means of a water bath. No attempt was made to screen out visible or other rays from the spectrum of the arc.) Dilutions of sperm were made after the manner of F. R. Lillie, '15*b*, on a percentage basis, using as "stock" the thick fresh sperm as it exuded from the genital pore of cut, inverted *Arbacia* males.

Immediately after exposure, samples of one drop each of radiated and nonradiated sperm of the same dilution, were transferred to dishes containing 10 cc. (300 drops) [1] of a known concentration of eggs in sea water (3–5 per cent. in these experiments). In drawing off samples of radiated sperm, care was taken to obtain sperm from the upper surface at the center of the dish. At short intervals thereafter, up to three hours, samples of radiated and nonradiated sperm, kept under similar conditions, were used to fertilize normal eggs. At the end of 4 or 5 hours, the percentage of eggs which had formed membranes and of those which had cleaved was determined on the basis of a count of 200 eggs in each case.

By comparing results obtained following ultraviolet radiation, with those obtained from normal lots of eggs it was possible to estimate the degree to which radiation had inhibited the fertilizing power of a given dilution of sperm.

Results.—It was found that in normal sperm suspensions, fertilizing power decreased as time after removal of the first sample increased; the more dilute the sperm suspension, the more rapid was the rate of decrease. (See also F. R. Lillie, '15*b*.) Time and dilution, then, are factors in determining the fertilizing power of normal sperm. (See Table I.)

Exposure of dilute sperm to ultraviolet radiation augments the rate of loss of fertilizing power beyond that following dilution alone. Fewer eggs cleave (see Table I.), or even form mem-

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[1] Sperm was thus further diluted 300 times. Concentrations of sperm at the time of insemination were therefore 1/300 per cent. to 1/72,000 per cent., ($\frac{1}{2}^{8}$ per cent. to $\frac{1}{2}^{16}$ per cent.)

TABLE I.

THE EFFECT OF RADIATION ON THE FERTILIZING POWER OF SPERM.[1]

Time (minutes).	*A.* Normal Nonradiated Sperm.									*B.* Radiated Sperm.								
	Dilution of Sperm (Powers of 2).																	
	8	9	10	11	12	13	14	15	16	8	9	10	11	12	13	14	15	16
	Percentage of Eggs Cleaved.									Percentage of Eggs Cleaved.								
0	100	99	100	100	99	90	95	73	36	97	88	91	68	49	24	22	8	2
3	100	—	98	—	—	76	—	—	14	92	—	84	—	—	11	—	—	0
5	100	99	98	100	98	73	94	54	5	97	96	72	55	36	7	8	6	.5
10	100	—	—	98	—	—	89	51	7	99	—	—	49	—	—	5	4	0
15	100	99	98	99	95	49	67	46	6	99	84	44	51	19	2	5	3	0
30	97	100	92	94	68	18	30	41	1	95	96	21	40	13	2	4	1	0
45	99	100	91	70	63	27	—	14	.5	93	94	30	42	5	3	—	0	0
60	99	100	92	65	49	19	30	11	.5	43	82	21	35	2	1	1	.5	0
90	94	96	28	54	28	17	15	2	0	25	89	11	22	1	1	0	0	0
120	94	—	29	38	21	3	7	2	—	5	—	2	15	.5	1	.5	0	—
150	—	94	—	—	9	—	1	.5	—	—	25	—	—	0	—	.5	0	—
180	88	—	12	27	2	3	2	.5	0	2	—	0	6	0	1	0	0	0

branes, in comparable lots of eggs fertilized by exposed sperm, as compared with eggs fertilized by normal sperm. Also, the rate at which fertilizing power is lost is greater in the more dilute suspensions. (See Fig. 1.) Bohn and Drzewina, '23, showed that the more dilute the sperm suspensions of *Arbacia* were, the more susceptible they were to the inhibiting influence of neutral red in the presence of light.

Figure 1 indicates that in general, radiation shifts the curve of the dilution effect to the left along the time axis. The greater the dilution, the more immediately is the rate of falling off augmented as compared with normal lots. For example, Fig. 1 gives the following data with respect to increase in the rate of falling off in the fertilizing power of *Arbacia* sperm. In dilution $\frac{1}{2}^{8}$ per cent. (Curve *A*), the increase in rate is not felt until one hour after exposure, but the continued decrease in fertilizing power lasts until three hours after exposure. In more dilute suspensions, $\frac{1}{2}^{10}$ per cent., $\frac{1}{2}^{12}$ per cent. (Curve *B*), $\frac{1}{2}^{14}$ per cent., the increase in rate is noticeable only up to one hour, $\frac{3}{4}$, and $\frac{1}{4}$ hours respectively. With still greater dilution, $\frac{1}{2}^{15}$ per cent.

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[1] Measured by the percentage of cleavage in normal eggs fertilized by treated sperm.

Figures 8–16 represent sperm dilutions of $\frac{1}{2}^{8}$–$\frac{1}{2}^{16}$%. (11 and 12 are averages of three experiments; 15 of two; and the rest, one each.)

Figures in the first column to the left represent the time in minutes following initial dilution and exposure to radiation.

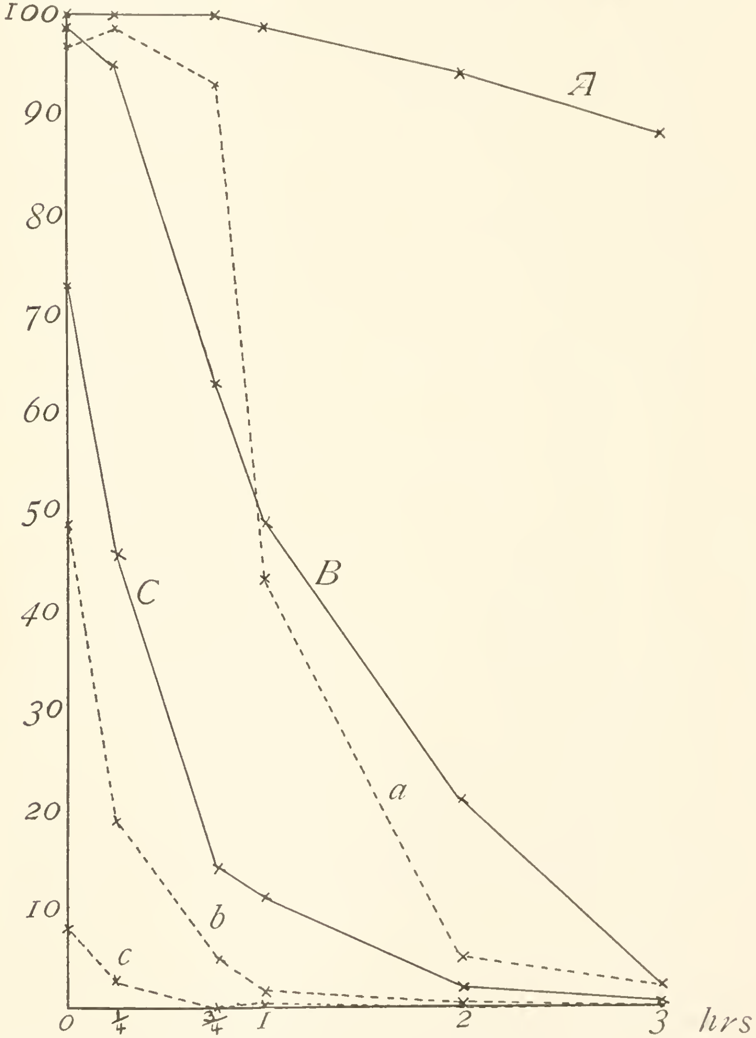

FIG. 1. The effect of radiation on the fertilizing power of sperm. Time and dilution are factors. The more dilute suspensions are more susceptible, particularly immediately after radiation. *A*, *B*, *C*—normal nonradiated sperm. *a*, *b*, *c*—radiated sperm in corresponding dilutions. *A*, *a*—$\frac{1}{2}^{8}$%; *B*, *b*—$\frac{1}{2}^{12}$%; *C*, *c*—$\frac{1}{2}^{15}$% sperm dilutions. Ordinates represent the percentage of eggs cleaved. Abscissæ represent the time in hours, since the beginning of the exposure of sperm to radiation.

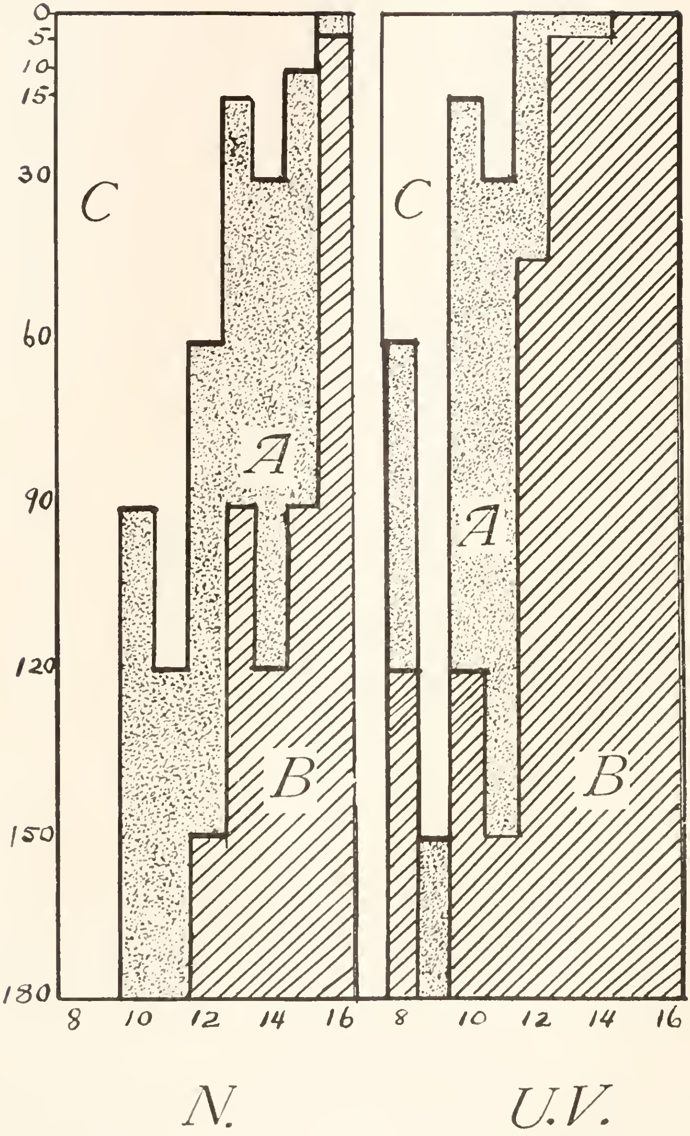

FIG. 2. Reduction of fertilizing power. Radiation reduces the time required to decrease the fertilizing power in all dilutions. Again, the more dilute suspensions are more susceptible. *N*—normal nonradiated sperm. U.V.—radiated sperm. *A*, *B*, *C*—represent percentages of eggs in early cleavage stages. *A*—a reduction of from 10 to 50% in the number of eggs which cleaved. *B*—a reduction of from 90% in the number of eggs which cleaved. *C*—a reduction of from 0 to 50% in the number of eggs which cleaved. 8–16 represent sperm dilutions, *e.g.*, $\frac{1}{2}^8$% to $\frac{1}{2}^{16}$%. Ordinates represent the time in minutes elapsed since the beginning of the experiment. Abscissæ represent the degree of sperm dilution.

(Curve *C*) to $\frac{1}{2}^{16}$ per cent. the increase in rate of loss of fertilizing power is immediate.

A study of Fig. 2 reveals the fact that it takes longer to reduce the fertilizing power below 50 per cent. in normal sperm suspensions than in radiated suspensions of comparable dilution; that more time is needed to bring about a 50 per cent. reduction in the less dilute suspensions; and that when normal eggs are fertilized by dilute radiated sperm ($\frac{1}{2}^{12}$ per cent. to $\frac{1}{2}^{14}$ per cent.), less than 50 per cent. cleavage follows; and finally that when sperm dilutions reach $\frac{1}{2}^{15}$ per cent. to $\frac{1}{2}^{16}$ per cent., less than 10 per cent. of the eggs cleave. (See Table II.) The figures indicate the number of minutes, following initial dilution and exposure of sperm suspensions, required to reduce cleavage 50 per cent. and 90 per cent. It will be seen that less time is required in dilute suspensions, indicating that they are more susceptible to radiation effects.

TABLE II.

THE EFFECT OF RADIATION OF SPERM ON THE PERCENTAGE OF CLEAVAGE OF NORMAL EGGS.

	A. Normal Nonradiated Sperm.									*B.* Radiated Sperm.								
	8	9	10	11	12	13	14	15	16	8	9	10	11	12	13	14	15	16
Time Required to Reduce Cleavage.[1]																		
50%	—	—	60	90	45	10	15	10	0	45	120	10	15	0	0	0	0	0
90%	—	—	—	—	120	60	90	60	5	90	120	90	150	30	10	5	0	0

That cleavage is retarded and abnormally modified by the radiation of sperm, and that the length of time elapsed after dilution determines the severity of the effect, even in nonradiated suspensions, is clearly shown. When sperm in the concentration of $\frac{1}{2}^{11}$ per cent. is radiated for 5 minutes and is then used at intervals to fertilize normal eggs, no larvæ are formed, and cleavage is abortive and reduced 90 per cent. or more. Table III. shows such a series for normal and radiated sperm in like concentrations.

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[1] Time in minutes, following dilution, required to bring the sperm into a state at which 50 or 90 per cent. of the eggs fail to cleave.

Figures 8–16 represent sperm dilutions of $\frac{1}{2}^{8}$–$\frac{1}{2}^{16}$ per cent.

TABLE III.

THE EFFECT OF RADIATION ON $\frac{1}{2}^{11}$ PER CENT. SPERM WHEN TIME IS ALSO A FACTOR.

(Sperm was radiated for five minutes, and immediately thereafter used to fertilize normal eggs.)

Time[1]	*A.* Normal Nonradiated Sperm.	*B.* Radiated Sperm.
0	100% swimming larvæ	No larvæ; less than 1% abortive cleavage
3	100% " "	" " 1% " "
5	99% " "	" " " " 1% " "
11	100% " "	" " 2% " "
15	100% " "	" " " " 1% " "
30	97% " (3% abnormal)	" " " " 1% " "
45	80% " (10% ")	" " 3% " "
60	50% " (30% ")	" " 7% " "
90	25% " (50% ")	" " 10% " "
150	5% " (75% ")	" " 1% " "

When examined five minutes after exposure, less than 1 per cent. of the radiated sperm show any motility. Later, however, after about nine hours the sperm may be seen to cluster about the eggs, indicating some degree of recovery of motile power. The recovery of motile power following inhibition by radiation, recalls the assertion made by Glaser, '14, namely, that paralyzed sperm may be reactivated but not reagglutinated.

Motility is considerably reduced soon after exposure to ultraviolet radiation (see also Lillie and Baskervill, '22). Sperm in the more dilute suspensions more quickly lose their power of movement, and clumping or agglutinating takes place early. The clumps are large and irregular and stable. The higher the dosage, the larger are the clumps of sperm. In viewing an exposed lot of sperm with a microscope, it can be seen that the surface layer. contains immobile sperm, while the layers below (as seen in optical section) have been "shaded" from the ultraviolet radiation (or have not been reached because of insufficient penetration of the rays), and are therefore normally active. The extreme bottom layer of the dish again contains inactive sperm which have probably sunk down from the top layer. Clusters of sperm are numerous. In transmitted light, radiated sperm suspensions appear "clear," while nonradiated ones appear "milky."

When sperm in dilutions ranging from 1/300 per cent. to

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[1] Time elapsed (in minutes) since exposure to radiation for 5 minutes.

1/240,000 per cent. is exposed to radiation from the mercury arc for periods of 2 and 5 minutes, it becomes evident that the more dilute suspensions are more susceptible to reduction in fertilizing power than the more concentrated suspensions. Nonradiated controls show 100 per cent. cleavage in all dilutions up to 1/30,000 per cent., in which case 60 per cent. of the eggs cleave normally, 15 per cent. abnormally, and 25 per cent. fail to cleave. When a dilution of 1/240,000 per cent. is reached the total cleavage amounts to but 9 per cent., when sperm are used immediately after radiation to fertilize normal eggs. Dilution of sperm is certainly a factor in modifying its fertilizing power, even in nonradiated suspensions.

Table IV. shows the effects of radiation on cleavage. It will be seen that membrane formation is possible, with less dilute suspensions of sperm when radiation does not exceed 2 minutes. That the degree of inhibitory effect of radiation increases with dosage is indicated here.

TABLE IV.

THE EFFECT OF RADIATION OF SPERM ON THE RATE OF CLEAVAGE OF EGGS.

(Eggs were fertilized immediately following dilution and radiation of sperm.)

	A. Sperm Radiated for 2 Minutes.									*B*. Sperm Radiated for 5 Minutes.								
	Dilution of Sperm (Powers of 2).																	
	8	9	10	11	12	14	15	17	18	8	9	10	11	12	14	15	17	18
A........	25	14	0	30	12	1	<1	—	—	5	2	—	15	<1	1	1	—	—
B........	6	8	10	2	<1	<1	—	<1	—	1	2	2	1	—	—	—	—	—
C........	60	3	5	1	<1	<1	—	—	—	—	1	—	—	—	—	—	—	—
D........	9	75	85	67	87	97	99	99	100	94	95	98	84	99	99	99	100	100

Dilutions as before. 11 and 15 are averages of three experiments each; 14 of two; and the rest, one each.

A—Advanced cleavage.	*C*—Membranes only.
B—Slow cleavage.	*D*—No membranes.

That the degree of the effect of radiation is roughly proportional to dosage is better shown in Table V., where the exposures were briefer. In these experiments, on the day following radiation of sperm, swimming preplutei were found in radiated lots, only with sperm dilutions of $\frac{1}{2}^{8}$ per cent.; early gastrulæ were plentiful in $\frac{1}{2}^{9}$–$\frac{1}{2}^{14}$ per cent., and scarce in $\frac{1}{2}^{15}$–$\frac{1}{2}^{18}$per cent. In the control series (nonradiated) all dilutions showed swimming preplutei.

The effect of ultraviolet radiation on fertilizing power is, in general, roughly proportional to the dosage, *e.g.*, exposures of 5 minutes are more effective in causing immediate reduction of fertilizing power than are exposures of a minute or less. Also,

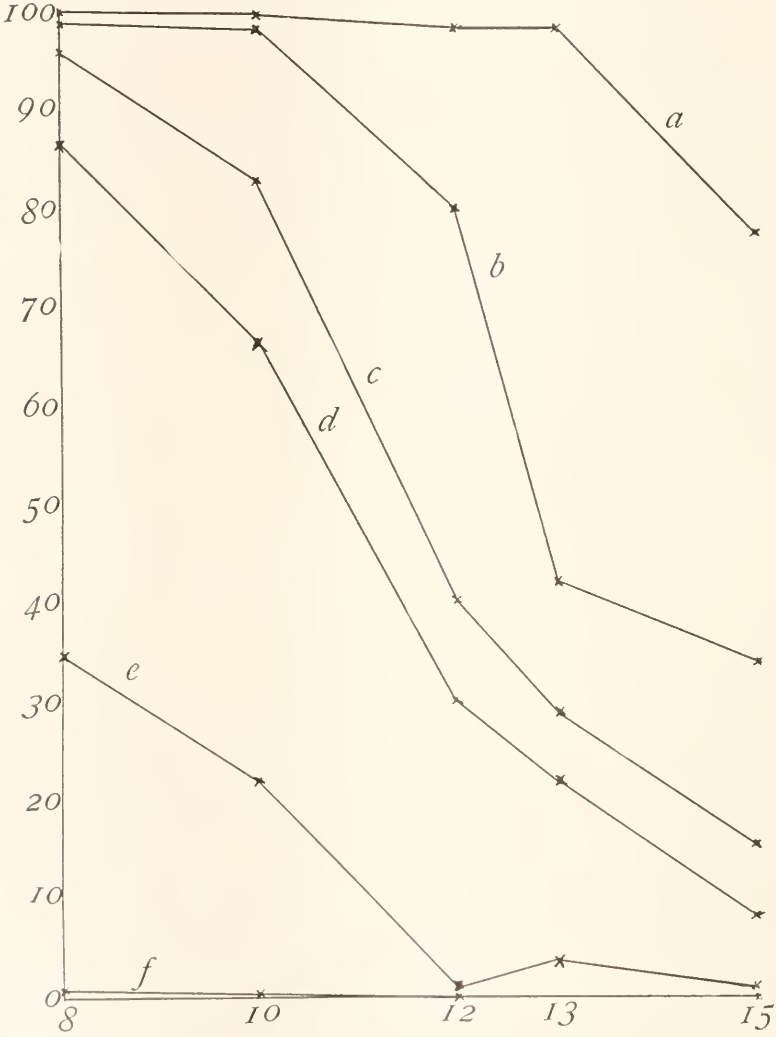

FIG. 3. The effect of length of period of exposure on fertilizing power. The degree of reduction is roughly proportional to the dosage of radiation. *a–f* represent the lengths of the periods of exposure in minutes. *a*—0, *b*—$\frac{1}{4}$, *c*—$\frac{1}{2}$, *d*—1, *e*—3, *f*—10. Ordinates represent the percentage of eggs cleaved. Abscissæ represent the dilutions of sperm, *e.g.*, 8—$\frac{1}{2}^{8}$%, etc.

TABLE V.

THE RELATION OF DOSAGE OF RADIATION TO THE DEGREE OF INHIBITION OF CLEAVAGE.

Dosage in min.	Dilution of Sperm (Powers of 2).				
	8	10	12	13	15
	Percentage of Eggs Cleaved.				
0	100	99.5	98	98	77
$\frac{1}{4}$	99	98	80	42	34
$\frac{1}{2}$	96	83	40	29	15
$\frac{3}{4}$	87	79	42	20	11
1	87	67	30	22	8
3	35	22	1.5	4	1.5
5	2	1	.5	1	.5
10	1	.5	0	0	0

Figures 8–15 represent sperm dilutions $\frac{1}{2}^{8}$–$\frac{1}{2}^{15}$ per cent.

the degree of reduction in each case is more marked in the weaker suspensions, *e.g.*, exposures of less than one minute reduce the fertilizing power 50 per cent. in dilutions of $\frac{1}{2}^{12}$ per cent. to $\frac{1}{2}^{15}$ per cent. The same exposure reduces the fertilizing power of dilutions of $\frac{1}{2}^{8}$ per cent. to $\frac{1}{2}^{10}$ per cent. only 10–20 per cent. Five and ten minute exposures reduce the fertilizing power to 2 per cent. or less in all dilutions. (See Figure 3.)

Discussion.—The loss of fertilizing power of normal sperm suspensions occasioned by dilution and staling is greatly accelerated by exposure to ultraviolet radiation. The greater the dilution of the sperm, the more rapid is the reduction of fertilizing power, and the more delayed and abnormal is the cleavage. Dosage of radiation is also a factor in determining the degree of inhibition of fertilizing power. Motility is reduced and the sperm forms aggregations which are large and irregular, and recall those described by F. R. Lillie, '12*b*, '13, '15*a*, Just, '19, J. Loeb, '14, and Sampson, '22. Exposure to ultraviolet radiation appears to increase the surface adhesiveness of sperm, inducing agglutination, a change probably accompanied by a change in permeability of the sperm membrane. (See also F. R. Lillie, '13, in this connection.)

Although motility is considerably interfered with, the loss of fertilizing power does not necessarily parallel the loss of motility.

(See F. R. Lillie and Just, '24, p. 134.) Exposures up to 5 minutes produce but a slight immediate inhibition of motility; exposures of 10–20 minutes produce a noticeable inhibition, while exposures of 30 minutes completely paralyze the sperm. However, even after so long an exposure, a small percentage of the eggs reach the 2- and 4-cell stage (less than 1 per cent.), but proceed no further, suggesting that normal fertilizing power is probably impaired before motility is lost. On the other hand, shorter exposures ($\frac{1}{4}$ min. to 1 min.) produce a marked reduction of fertilizing power as measured by the percentage of eggs which produce membranes and cleave.

It seems probable that the loss of fertilizing power is due rather to the outward diffusion of some substance necessary for fertilization. Such a substance, if present at or near the surface of the sperm, may easily diffuse into the medium by changes induced in the sperm surface by surface-acting radiation. This would result in the disturbance of the normal concentration equilibrium of such a substance in the sperm which would then become incapable of inducing normal and complete fertilization, since the latter probably depends on an optimum concentration of such a substance within the sperm.

As before stated, highly diluted sperm suspensions are more susceptible to injury than are the more concentrated suspensions. Diffusion of sperm substance to the external medium following a change in the permeability of the sperm surface induced by radiation, would proceed more rapidly in dilute than in concentrated suspensions, since fewer sperm per unit volume of suspension would contribute to the establishing of an external-internal equilibrium. Diffusion of a substance necessary for fertilization, from the sperm into the medium may explain the loss of fertilizing power induced by dilution and long standing. It may also explain why more dilute suspensions are more susceptible to an influence which is additive in its action on the diffusion rate of such a substance.[1]

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[1] Compare the mass effects obtained by Robertson, '22, Drzewina and Bohn, '21, '22. See also a later paper by Drzewina and Bohn, '23*a*, for suggestion of mass effect on sperm. Glaser's suggestion (Glaser, '15) of the necessity for the presence of more than one sperm per egg in order to insure successful fertilization, may have some bearing on the problem of mass effects in sperm.

When fertilization is incomplete or partial, abnormal cleavage and abnormal development follow. (See Table IV; also previous paper, Hinrichs, '26*b*.) F. R. Lillie, '11, '12*a*, '19, in analyzing the fertilization reaction in *Nereis*, divided it into two phases, external and internal. The former concerns itself with membrane formation and needs only the contact of sperm for its initiation. Other investigators have obtained similar results; thus, Just, '19, found that although sperm entry may initiate cortical changes, it is no criterion of fertilization, and further development does not necessarily follow. Loeb, '13, states that cortical changes in the sea urchin are not dependent on the penetration of sperm. Dungay, '13, found that injury of sperm may prevent its entry into the egg, and maturation may go on, but cleavage fail to follow. (See also the recent paper by O. Hyman, '25.)

In the experiments with ultraviolet radiation, membrane formation is usually less interfered with than cleavage. There may be an advance of from .5–5 per cent. in the number of eggs having membranes only, over those cleaved. This seems to be an indication that the fertilization reaction was initiated but not completed.

Complete internal fertilization involves compatibility of germinal nuclear material, and forms the basis for normal development. When either sex component has been injured, *e.g.*, by ultraviolet radiation, development becomes abnormal, particularly in the regions of the organism which normally have a relatively high rate of physiological activity. (See also Hinrichs, '26*b*.) Such a result is comparable to that reported by Newman, '17, for teleost hybrids, where incomplete fertilization resulting from the incompatibility of germ plasms produces differentially modified embryos.

It would be difficult to explain so complete a series of differentially inhibited forms as those shown in Plates I. and II. of a previous paper (Hinrichs, '26*b*) on the assumption of parthenogenetic development of the egg induced by injury to the sperm nuclear material. Also, the fact that the degree of effect (as measured by the percentage of eggs cleaved and the type of cleavage) is proportional to the dosage of radiation to which the sperm is subjected, can hardly be satisfactorily explained in this way.

Cohn, '17, '18, has shown that sperm in dilute suspensions are more active than in concentrated suspensions. Active cells are, on the whole, more susceptible to injury by radiation than are quiescent cells. (See Hinrichs, '24, '26*b*.)

Ultraviolet radiation is particularly suited for experiments of this kind, as compared with chemical agents, because,

(1) Exposures may be very short and still be effective. A superposition of the effect of staling is thus impossible.

(2) The possibility of carrying over into the normal egg, at the time of fertilization, small amounts of injurious reagent is absent. Results are clear-cut and more likely to be due to ultraviolet radiation alone, uncomplicated by other factors.

I wish here to express my thanks to Dr. R. S. Lillie for his interest and help in the study of this problem. .

Conclusions.—(1) Further data are given on the normal loss of fertilizing power in sperm suspensions due to dilution and staling. These results are entirely in accord with the ones reported by F. R. Lillie in 1915.

(2) Ultraviolet radiation augments the rate of loss of fertilizing power beyond that produced by time and dilution.

(3) Dilute sperm suspensions lose their power of fertilization earlier and more rapidly when radiated (and even without radiation) than do the more concentrated suspensions.

(4) The rate of loss of fertilizing power is roughly proportional to the dosage of radiation.

(5) The motility of sperm is impaired and cleavage is delayed and abnormal. Development is differentially modified. (See previous paper, Hinrichs, '26*b*.)

(6) Ultraviolet radiation produces sperm agglutination.

(7) Fertilizing power decreases more rapidly than does motility in both radiated and nonradiated suspensions.

(8) Fertilization is incomplete when normal eggs are fertilized by radiated sperm, and may in some cases lead only to membrane formation.

(9) The loss of fertilizing power is probably due to the loss from the sperm, by outward diffusion, of some substance necessary for fertilization. Ultraviolet radiation augments the loss of fertilizing power, presumably by altering the surface of the

sperm, and thereby increasing its permeability to such a substance.

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THE SEASONAL INFESTATION OF *NASSA OBSOLETA* (SAY) WITH LARVAL TREMATODES.

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The purpose of this investigation has been to make a twelve months' survey of large numbers of a single species of marine mollusk, to determine what different larval trematodes parasitize it, and especially to determine and to try to interpret the seasonal fluctuations in the degree and character of the infestation. The study was begun at the Marine Biological Laboratory, Woods Hole, Massachusetts, in August, 1924, when several common species of gastropod were collected in Quamquisset Harbor. Examination at that time showed *Nassa obsoleta*, the common mud snail, to be the most heavily parasitized, and therefore this species was chosen as the one for study throughout the year. The biology and ecology of this snail have been studied by a number of investigators (see especially Dimon, 1905; Sumner, Osburn, and Cole, 1913; Allee, 1923*a*, 1923*b*). The work was continued at the Zoölogical Laboratory of Washington University with snails shipped periodically from Woods Hole, and the twelve months' survey was completed at Woods Hole during the summer of 1925. All collections were made from a fifty yard area in the part of Quamquisset Harbor known as Gansett; a total of 8,875 individuals of *Nassa obsoleta* were examined. In addition to the data on the seasonal infestation brief descriptions of the larval trematodes are also included.

HISTORICAL.

Relatively little work has been done on marine larval trematodes, and that chiefly by European investigators. Among these Pelseneer (1906), Sinitsin (1911), and Lebour (1905–1912) have contributed most extensively. There are only a few scattered references on North American cercariæ. Fewkes (1882) briefly described a cercaria with caudal setæ found free near Newport, Rhode Island. Tennent (1906, 1909), worked out the life history of *Bucephalus haimeanus* and described its gasterostome cercaria. Linton (1915*a*, 1915*b*) found three species of cercariæ in the Woods Hole region: two furcocercous forms, one from *Hydroides dianthus* and one from *Pecten irradians*, and one tailless larva from *Nassa obsoleta* which has been designated *Cercariæum lintoni* in this paper. More recently one of us (Miller, 1925*a*, 1925*b*) has made surveys of the larval trematodes infesting marine gastropods from Puget Sound and from the Dry Tortugas.

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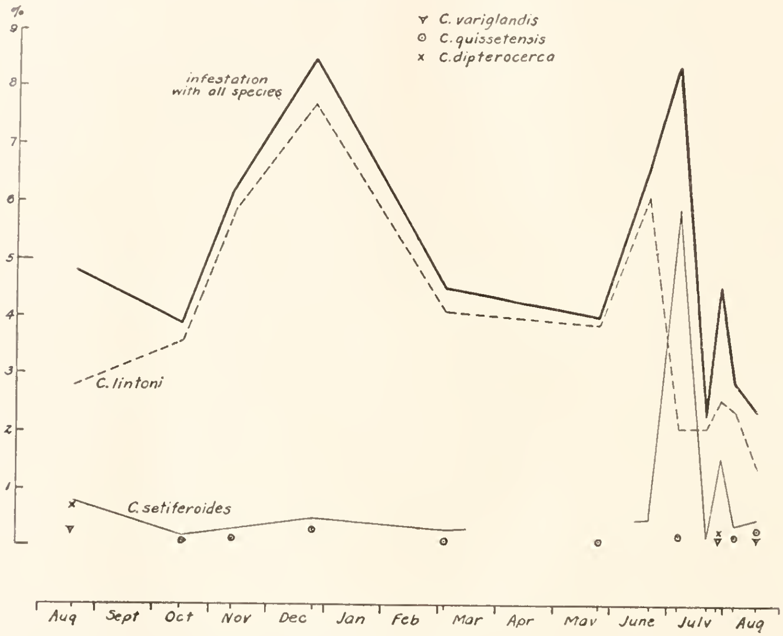
[1] The routine examination of snails and collection of data for descriptions of the trematodes are almost wholly the work of the junior author, as are all drawings except numbers 2, 9, 11 and 17.

MATERIAL AND METHODS.

Snails received at Saint Louis were maintained until used in synthetic sea water made up according to the formula of Ditmar. All individuals of a collection, except that of May, were isolated for forty-eight hours to determine those from which mature larvæ were emerging. These infested snails were placed in separate aquaria as a constant source of living cercariæ for study. At convenient times the remaining snails were crushed individually, and their tissues examined under a binocular dissecting microscope in order to find non-emerging cercariæ and their parthenitæ; these were usually present in the digestive gland. After the living material had been studied, the infested viscera, freed cercariæ, and parthenitæ were fixed in Bouin's, or hot corrosive sublimate fluid. Cercariæ and parthenitæ were stained with Ehrlich's acid hematoxylin and mounted in Canada balsam. Unless otherwise noted, all measurements recorded were taken from these preserved specimens. Sections of snail viscera, two and one half and five micra in thickness, were variously stained.

SEASONAL INFESTATION.

The graph for total infestation (Text-fig. 1) with all five species of larvæ has two maxima, practically equal, occurring in December and in July. Between these maxima are low areas, in each of which the percentage of infestation averages not more than one half that of the maximum. This plainly shows a seasonal fluctuation in the presence of larval trematodes in this particular host. Before discussing this, the frequence and nature of the infestation with each of the five larvæ found will be taken up. Two of them were present only infrequently and in low percentages, and may be dismissed with brief statements. *Cercaria dipterocerca* sp. nov. was found in only four specimens, three in August, 1924, and one in late July, 1925; and similarly, *C. variglandis* sp. nov. was found in one host in each of these same collections, and once in August, 1925. There is no obvious explanation for the slight and infrequent occurrence of these two trematodes in *Nassa obsoleta*. It may be that they are normally parasitic in some other mollusk host, but are capable of developing occasionally in this species of *Nassa;* or the final hosts may be migratory.



TEXT-FIGURE I. Graphs showing seasonal fluctuation in total infestation of *Nassa obsoleta*, and infestation with each species of trematode.

A third species, *C. quissetensis* sp. nov., was found in eight of the twelve collections, always in very few hosts, and never emerging after isolation of the snail. In the October collection the rediæ were filled with mature cercariæ; in November and

December only immature rediæ were present, while in the succeeding months there were always some apparently mature, but non-emerging cercariæ. In July and August, 1925, many fully developed cercariæ together with a few early germ balls were present in all of the rediæ. A fourth species, *C. setiferoides* sp. nov. was found in every collection but that of May. Although usually less than one per cent. of *Nassa* was infested, in early July (1925) 5.9 out of a total of 8.4 per cent. infestation was due to this species. Some mature, or at least apparently fully formed, cercariæ were found in every collection, which would seem to indicate that *Nassa obsoleta* is continually being infested by the miracidia of this trematode. There was no emergence of *C. setiferoides* from isolated snails until June and July, 1925; this seems to be the season of maturity of this larva, although some infestations with immature rediæ were found both in late July and in August, 1925.

Much the greatest part of the total infestation was due to the presence of a tailless larva, *Cercariæum lintoni* sp. nov., which Linton (1915*b*) briefly described but did not name. It was present in every collection, and except in that of July 6, 1925, the percentage of infestation with this species was greater than the total for all other trematodes present (Text-fig. 1). Although fully developed larvæ were found throughout the year, there was a striking seasonal variation in the percentage of snails infested with mature[1] larvæ. In Text-figure 2, the total percentage of infestation of *Nassa obsoleta* with *Cercariæum lintoni* and the percentage of snails from which mature larvæ emerged are shown graphically. The two maxima of the total infestation graph might be ascribed to the semi-annual visitation of Woods Hole by the definitive vertebrate hosts, whether migratory birds or fishes. It is presumed that the majority of the definitive hosts of marine cercariæ are fishes, concerning the migrations of which relatively little is known (Meek, 1916; Bigelow and Welsh, 1925).

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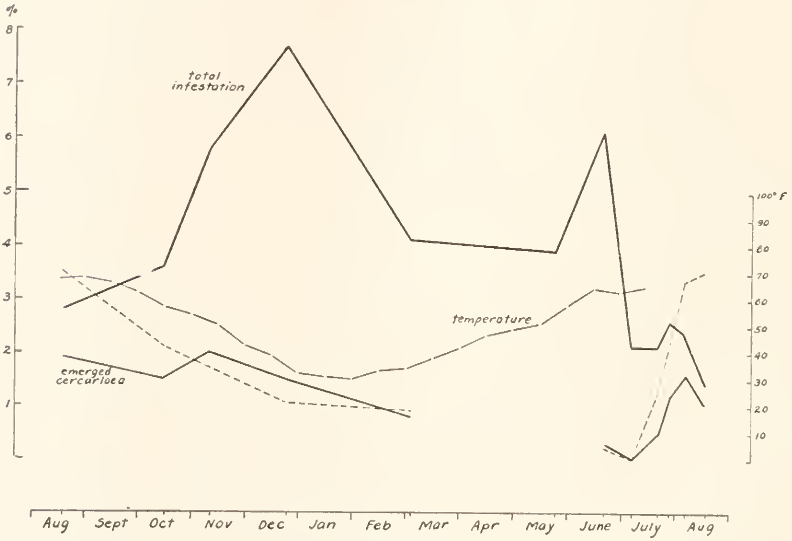
[1] The emergence of fully formed larvæ from the snail when isolated in sea water for forty-eight hours is taken as evidence of the full maturity of the trematode. In some cases the larvæ in crushed snails were apparently fully formed, but did not emerge, for reasons unknown. Whether or not emergence is a fair test of maturity, the same procedure has been followed with all collections (except the May shipment, when they were not isolated due to lack of time). It is possible that in the case of some snails only a few larvæ emerged and that these escaped observation.

Why a certain percentage of infestation, such as the maximum of December or June, once reached is not maintained would seem to be due directly to two factors, the death of heavily infested snails, and the recovery of some of the snails because of the maturing and complete emergence of the larval trematodes. The serious and often fatal effects of larval trematode parasites on the snail host have been studied by a number of investigators.



TEXT-FIGURE 2. *Cercariæum lintoni;* graphs showing total infestation and percentage of emerged (mature) cercariæ.

The very rapid increase in actively metabolizing trematode tissue, which frequently results in the destruction of most of the visceral mass of the snail, probably causes the death of large numbers of them. As to recovery, Sewell (1922: 17) and others have noted a condition of degeneration of liver or gonad apparently attributable to previous trematode infestation; the present author has also found this condition in some individuals of each of a number of species, such that at first glance the liver appeared to be parasitized, but no trematodes were present. Sewell included also the factor of natural death at certain seasons of the year as one which might explain seasonal fluctuations in percentage of infestation. This operates, according to him, to raise the percentage of infestation by removing old snails which had formerly been infested and had recovered and become immune. He believes that the majority of fresh water mollusks may die from natural causes in June and July. This is borne out by the observations of Manson-Bahr and Fairley, Annandale, and others. This factor is probably an important one among the fresh water snails of India which Sewell studied, for the percentage of infestation was frequently very high and the number of old, immune individuals was probably relatively quite large. But among marine gastropods, where usually only a small percentage harbors larval trematodes, it would not be expected that this factor

TABLE I.

INFESTATION RECORD OF 8,875 SPECIMENS OF *Nassa obsoleta*.

Date.	Number Collected.	Died before Examination.	Total Per Cent. of Infestation.	Infestations.	Per Cent. of Infestation.
8/17/24	353	0	4.8	10, *C. lintoni* (7, emerged). 3, *C. setiferoides* (2, immature rediæ only). 3, *C. dipterocerca* (1, emerged). 1, *C. variglandis*.	2.8 0.8 0.8 0.3
10/14/24	869	2	3.9	31, *C. lintoni* (13, emerged). 2, *C. setiferoides* (1, immature rediæ only). 1, *C. quissetensis*.	3.6 0.2 0.1
11/11/24	995	3	6.2	58, *C. lintoni* (20, emerged). 3, *C. setiferoides* (2, immature rediæ only). 1, *C. quissetensis* (immature rediæ only).	5.8 0.3 0.1
12/24/24	947	9	8.5	73, *C. lintoni* (15, emerged). 5, *C. setiferoides* (2, immature rediæ only). 3, *C. quissetensis* (immature rediæ only).	7.7 0.5 0.3
3/3/25	1,153	9	4.5	47, *C. lintoni* (9, emerged). 4, *C. setiferoides*. 1, *C. quissetensis*.	4.1 0.3 0.1
5/22/25	727[1]	8	4.1	29, *C. lintoni*. 1, *C. quissetensis*.	4.0 0.1

[1] Not isolated for emergence.

TABLE I.—(*Continued*).

Date.	Number Collected.	Died before Examination.	Total Per Cent. of Infestation.	Infestations.	Per Cent. of Infestation.
6/20/25	649	1	6.6	40, *C. lintoni* (2, emerged). 3, *C. setiferoides* (1, emerged).	6.2 0.5
7/6/25	509	0	8.4	11, *C. lintoni* (0, emerged). 31, *C. setiferoides* (7, emerged). 1, *C. quissetensis*.	2.1 5.9 0.2
7/21/25	575	0	2.3	12, *C. lintoni* (3, emerged). 1, *C. setiferoides*.	2.1 0.2
7/29/25	504	0	4.6	13, *C. lintoni* (6, emerged). 8, *C. setiferoides* (3, emerged, 4, immature rediæ only). 1, *C. dipterocerca*. 1, *C. variglandis*.	2.5 1.6 0.2 0.2
8/ 6/25	546	0	2.9	13, *C. lintoni* (9, emerged).	2.4
8/15/25	423[2]	0	0.7	3, *C. lintoni* (2, emerged).	0.7
8/18/25	625	0	2.4	9, *C. lintoni* (6, emerged). 3, *C. setiferoides* (very immature). 2, *C. quissetensis*. 1, *C. variglandis*.	1.4 0.5 0.3 0.2
Total. . .	8,875				

would play a considerable part. There are no data in Dimon's (1905) study of the biology of *Nassa obsoleta* on the life span of this species, nor whether large numbers die at any particular season of the year. The question of age immunity does not enter into the present study, as only data for large specimens of *Nassa* were included in the graphs; one collection of small individuals showed an infestation of only 0.7 per cent. on August 15, 1925, in contrast to 2.4 per cent. (Aug. 6) and 1.4 per cent. (Aug. 18) for two collections of large ones.

Inspection of the graph for emerged cercariæa (Text-fig. 2) shows that in the different collections there is variation in the percentage of snails harboring mature larvæ. There does not seem to be any correlation between these percentages and the fluctuations in the total percentage of infestation; the relative percentage of mature larvæ to total infestation is shown by the dotted line. It is unfortunate that the May collection was not tested for emergence; but from the available data it is seen that the occurrence of mature larvæ continues high from August to December, and then apparently is lower until late July and August, at which time it approximates its former values. The high percentages of emerged *C. lintoni* in July and August, 1925, are supported by the fact that during these months most of the sporocysts from crushed snails contained fully formed larvæ. Plotting of the temperature readings taken at the Fish Commission Wharf,[1] probably only in a general way comparable to that at Quamquisset Harbor, seems to show a relation between maturity and temperature; the maturity graph apparently lags behind that of temperature, remaining high while the temperature is dropping in November and December, and low for a considerable period after the temperature has increased in the spring and early summer months. Other factors which have already been discussed as affecting total infestation might also affect the percentage of mature cercariæa.

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[2] Small snails; data not included in any graph.

It is interesting to note that there is a close general similarity between the graphs shown by Sewell (1922: Chart 1) for two species of fresh water snails from India and the total infestation graph for *Nassa obsoleta* in the present study. The maxima for Sewell's examination of *Melanoides tuberculatus* from the Museum tank, fall in July and December, as they do for *Nassa obsoleta*, and the partial graph for *M. tuberculatus* from the Zoölogical Gardens follows the same general trend, with summer maximum in August rather than in July. Sewell's two graphs are based on 139 and 53 mollusk individuals respectively, while in the present study 8,452 large, and 423 small, specimens of *Nassa obsoleta* were examined.

[1] Temperature data were secured from the records of the Bureau of Fisheries for the 1st and 15th of each month of the period from August, 1924, to August, 1925; the figures are the mean of three daily readings taken at the U. S. Fisheries Station at 8 A.M., 12 M., and 4 P.M. The highest is 71° F. for August 1, 1924, and the lowest 30° F., just six months later, February 1, 1925. Thus there was at least an annual range of 41° Fahrenheit.

SUMMARY.

From the data resulting from the examination of almost nine thousand specimens of *Nassa obsoleta* Say it seems clear that there is a semi-annual rise and fall in the larval trematode infestation. In view of the fact that none of the adults of these larvæ are known it is difficult to explain these phenomena. In all probability migrations of the definitive hosts, and the degree of their infestation, affect the seasonal fluctuations; and other factors are probably the life span of *Nassa*, and the effect of parasitism upon it. The relative importance of these factors is not clear.

BRIEF DESCRIPTION OF NEW SPECIES CITED IN THIS PAPER.

CERCARIA SETIFEROIDES sp. nov.

(Figs. 1–3, 6, 10.)

Trichocercous distome cercaria with opaque yellowish body and characteristic excretory vesicle. Contractile body varying from 140 μ to 268 μ in length and 104 μ to 156 μ in width; average length 187 μ and width 126 μ; average tail length 486 μ. Oral sucker slightly elongate, 64 μ in average length and 34 μ in width; ventral sucker smaller and oval, 40 μ in width and 34 μ in length. Surface of body finely pebbled, and completely covered with short, regularly arranged spines. Conspicuous pigmented eye spots, composed of large spherical granules and a so-called lens. No spines on tail, but on either side of it rows of long setæ; usually seven setæ per group, the longest in the center; about thirty pairs of setæ groups arranged along sides of tail opposite to each other. Well developed digestive system clearly seen in both living and preserved material; mouth antero-ventral in oral sucker; short prepharynx followed by large oval pharynx, 24 μ in length and 16 μ in width; short esophagus, and two wide intestinal ceca extending to posterior end of body; jelly-like contents of ceca with great affinity for intra-vitam neutral red, in contrast to all other structures in body. Eleven pairs of larval glands, preacetabular in position, arranged in three groups; no observable ducts from the most posterior six glands; glands of all groups strongly acidophilic in all combinations of stains;

lightly stained with intra-vitam neutral red, but not with toluidine blue. Large excretory vesicle, the most conspicuous of internal structures, slightly to right of median line normally, and extending nearly to region of bifurcation of gut; very wide and bent more or less in shape of Z; filled with spherical refractile concretions, ranging from very small ones up to 3 μ in diameter. Main lateral collecting tube of either side entering excretory vesicle at a point about two thirds of distance from anterior end; entrance of anterior and posterior branches posterior to level of ventral sucker. Succession of single excretory flagella in wall of main lateral collecting tube; many flame cells observed, but exact pattern of excretory system not solved; apparently more highly developed posteriorly. Several cell masses present in anlage of reproductive system; no interpretation ventured as to parts of adult system represented. Development of cercariæ in rediæ in visceral mass of *Nassa obsoleta*. Immature rediæ slender, without locomotor appendages; average length 440 μ, and diameter at widest part 74 μ; long coiled gut extending beyond middle of redia. Germinal epithelium localized in posterior end. More mature rediæ with both mature cercariæ and germ balls.

C. setiferoides is similar to *C. setifera* Joh. Müller 1850 (described by Monticelli, 1914, and redescribed by Odhner, 1914, from one of Monticelli's slides), but differs in a number of important structures. It is obviously different from *C. lutea* Giard 1897, *C. pectinata* Huet 1891, and *C. setifera* Pelseneer, 1906; *C. fascicularis* Villot 1875 is not completely described. The cercaria with caudal setæ which Fewkes (1882) found free near Newport, R. I., may be identical with *C. setiferoides;* a detailed description of it was not published. The present species may also be identical with the larval form of *Pharyngora bacillaris*, recorded by Nicoll (1910) and Lebour (1917) as free in the plankton from Plymouth. The latter investigator (1916, 1917) found the metacercarial stage in various medusæ and in *Sagitta bipunctata;* her brief description and single figure of the trichocercous cercaria (1917) are not sufficiently detailed to determine whether it and the present species are the same.

CERCARIA DIPTEROCERCA sp. nov.

(Figs. 4, 5, 7, 8.)

Distome larva with prominent lateral cuticular fins extending along entire length of tail. Average body length 145 μ, varying between 109 μ and 212 μ, width 50 μ, varying between 47 μ and 65 μ. Body, but not tail, uniformly covered with small spines; double row of large ones around mouth. Pigment present only in two large eye spots, composed of very coarse granules and a "lens." Oral sucker 24 μ in diameter, larger than ventral sucker; subterminal mouth, prepharynx, pear-shaped pharynx; ceca not seen posterior to ventral sucker. Nine pairs of three different kinds of larval glands: on either side two pairs (Fig. 7, *a*), dorsal, lateral to pharynx, with coarsely granular, yellowish cytoplasm; stain with intra-vitam neutral red; eosinophilic in sections; second group of four pairs (*b*), laterally situated, with finely granular, greyish cytoplasm; basophilic with Ehrlich's acid hematoxylin; third group of three pairs (*c*), not observed in living cercaria; slightly basophilic in sections; no ducts found. Large thick walled excretory vesicle, varying from elongate oval to triangular in outline; main lateral collecting tubes and positions of some flame cells shown. Reproductive system represented by conspicuous mass of cells dorsal to ventral sucker. Tail with a pair of lateral convoluted cuticular fins extending the entire length; smaller median fin on ventral side of distal fourth of tail, extending around end up on to dorsal side; maximum tail length 320 μ, average 250 μ. Development of cercaria within elongate rediæ, averaging 795 μ in length and 92 μ in diameter; pharynx large; more or less cylindrical redia constricted at irregular intervals.

Cercaria dipterocerca differs from *C. hymenocerca* Villot 1875, *C. quadripterygia* Sinitsin 1911, and *C. lophocerca* (in Lebour, 1912) in significant details of structure.

CERCARIA VARIGLANDIS spec. nov.

(Figs. 15–17.)

Binoculate furcocercous distome cercaria most closely resembling members of the Elvæ group (Miller, 1923:44). Average body length 262 μ and width 77 μ; tail approximately same length as body; ratio of tail stem to furcæ about 3 : 2. Anterior penetrating organ, a highly modified oral sucker, 51 μ in greatest diameter; ventral sucker much smaller. Eosinophilic head gland observed in sections of anterior organ; ventral capillary mouth and esophagus as in other members of group. Body covered with short spines uniformly distributed; absent from tail and furcæ. Two eye spots composed of large pigment granules, in posterior connection with nervous system. Most of body filled with three differentiated sets of larval penetration glands: one pair of cells, posterior to eye spots, with finely granular cytoplasm, chromophobic in sections; two pairs of glands, dorsal to ventral sucker, with coarsely granular eosinophilic cytoplasm; three pairs of glands in posterior part of body, with rod-filled cytoplasm, homogeneous in sections, staining deeply with iron hematoxylin. No rapid selection of intra-vitam neutral red or toluidine blue shown by any set; anterior pair deeply stained, the two middle pairs chromophobic, and the three posterior pairs lightly stained in very strong solution of either dye. Five pairs of flame cells in body and one pair in proximal tail stem; exact connections of two posterior body flame cells not determined, but judging from fresh water larvæ for which excretory system is known pattern is probably like that of *C. wardi* (Miller, 1923: Text-fig. 4); excretory system in tail opening at tips of furcæ. Cell mass representing future reproductive system ventral and posterior to ventral sucker. Development of cercaria within long sporocysts of uniform diameter throughout, measuring on average 1.3 mm. in length and 0.37 mm. in width and containing between twenty and thirty cercariæ.

Cercaria variglandis is different in a number of respects from the few described marine furcocercous larvæ: *C. dichotoma* Müller 1850, *C. discursata* Sinitsin 1911, and *C. syndosmyæ* Pelseneer 1906.

Cercaria quissetensis sp. nov.

(Figs. 9, 11–14, 18, 19.)

Echinostome cercaria with twenty-seven spines on collar. Body averaging 290 μ in length and 130 μ in width (extreme extension of living cercaria 630 μ); tail about 330 μ in length. Oral sucker 42 μ, ventral sucker 64 μ in diameter. Mouth subterminal, short prepharynx, pharynx, wide esophagus bifurcating into ceca extending to extreme posterior part of body. Granular cytoplasm of small glands around esophagus and portions of contents of esophagus and ceca stained deeply with neutral red; dorsal gland cells eosinophilic and ventral ones chromophobic in sections; fine bundles of minute ducts passing through oral sucker and opening on anterior end. Many rod-filled cystogenous glands[1] in dorsal part of body; stained deeply with iron hematoxylin in sections. Excretory vesicle averaging 36 μ in diameter; arms filled with refractile concretions, of double coffee bean shape. Excretory system pattern not completely worked out; of type of *C. complexa* Faust (1919); twelve to fourteen flame cells observed on one side. Irregular masses of reproductive system cells posteriorly located, with a row of nuclei extending to a small mass anterior to ventral sucker. Average length of sausage-shaped redia 1.1 mm., width 0.2 mm.; orange-yellow pigment in wall; birth pore and two posterior locomotor appendages observed only in immature rediæ. Cercariæ encyst readily on glass slide, cysts averaging 142 μ in diameter, with two diametrically opposite projections.

Cercaria quissetensis differs in a number of respects from the six species reviewed by Lebour (1912), and from *C. proxima* and *C. sagitata* Lespès.

CERCARIEUM LINTONI sp. nov.

(Figs. 20, 21.)

Tailless larva, properly designated Cercariæum; original description by Linton (1915*b*), supplemented and emended in a few details by this study. Average length of a number of emerged larvæ, killed without pressure, 230 μ, width 84 μ. Very narrow intestinal ceca, traceable only in serial sections, reaching almost to excretory vesicle. One pair of prominent larval gland ducts, with granular contents, on either side of body, mistaken by Linton for excretory vesicles; four large eosinophilic larval glands, staining also with intra-vitam neutral red, but clearly observable only in sections. Large excretory vesicle, much constricted posteriorly, and surrounded by a sphincter muscle; opening in center of adhesive disc. A few flame cells observed, but exact pattern of excretory system obscured by numerous refractile globules distributed throughout body. Future reproductive system represented by a number of cell groups; two spherical masses, just posterior to ventral sucker, probably testes, with female complex anterior to excretory vesicle. Inchworm locomotion of this species effected by successive attachment of posterior end of body, modified by invagination into an adhesive disc, then extension of body and attachment by ventral sucker.

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[1] There is a possibility that the October and November infestations represent a second species of echinostome. The second infestation consisted of immature rediæ only, but in October the rediæ were filled with apparently fully formed cercariæ (Fig. 12). These differ from the mature cercariæ of the succeeding infestations chiefly in that they are of somewhat smaller size and different shape (Fig. 13), and lack cystogenous material. This is not considered as sufficient to differentiate them into two species.

Cercariæum lintoni is obviously different from *C. dentali*, *C. giardi*, and *C. crispata* of Pelseneer (1906), and from the five species described by Lebour (1912) in character of the digestive and excretory systems, or in the parthenita.

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PLATE I.

All drawings were made with the aid of a camera lucida, except where otherwise stated.

FIG. 1. *Cercaria setiferoides;* general view. × 150.

FIG. 2. Free-hand diagram of excretory system pattern in redia of *C. setiferoides.* × 40.

FIG. 3. Immature redia of *C. setiferoides*, showing pharynx and gut. × 92.

FIG. 4. *C. dipterocerca;* immature redia. × 88.

FIG. 5. General view of *C. dipterocerca.* × 56.

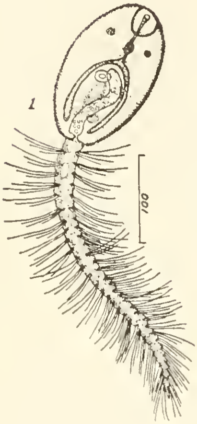
FIG. 6. Nearly mature redia of *C. setiferoides*, containing germ balls and almost fully formed cercariæ. × 72.

FIG. 7. *C. dipterocerca;* dorsal view of body showing eye spots, digestive and gland systems, and locations of most easily observable flame cells. Reproductive system cell mass between ventral sucker and excretory vesicle. × 415.

FIG. 8. Nearly mature redia of *C. dipterocerca.* × 138.

FIG. 9. Outline of body of *C. quissetensis*, when in extreme extension. × 42.

FIG. 10. *C. setiferoides;* dorsal view of body showing eye spots, digestive and gland systems, and cell masses representing future reproductive system. Only excretory vesicle and entrance of main lateral collecting vessels are shown. × 342.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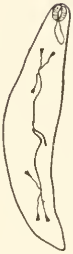
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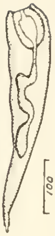
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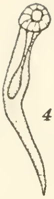
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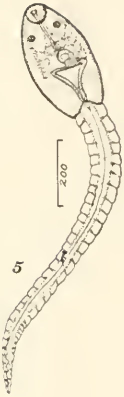
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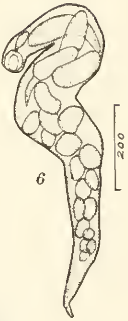
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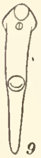
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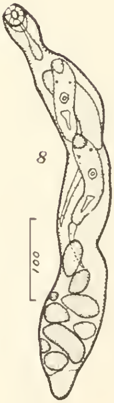
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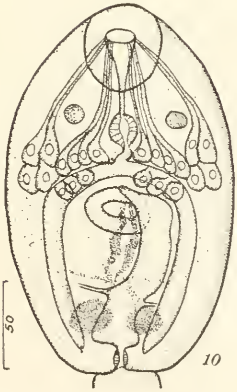
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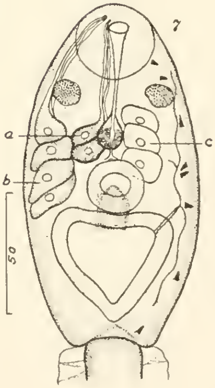
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Plate II.

Fig. 11. *C. quissetensis;* redia with gut and birthpore, containing only one fully formed cercaria. × 53.

Fig. 12. Redia of *C. quissetensis*, packed with mature cercariæ. × 47.

Fig. 13. Ventral view of body of *C. quissetensis;* digestive and gland systems; main trunks of excretory system; cell masses of future reproductive system. × 162.

Fig. 14. General view of *C. quissetensis*. × 93.

Fig. 15. *C. variglandis;* dorsal view showing anterior organ (highly modified oral sucker), eye spots, and three differentiated sets of gland cells. × 120.

Fig. 16. Sagittal section of body of *C. variglandis*, showing especially the different gland cells. × 385.

Fig. 17. *C. variglandis;* free-hand diagram of flame cell pattern in body. × 190.

Fig. 18. *C. quissetensis;* immature redia, showing pharynx and gut, and posterior locomotor appendages (on one side only). × 83.

Fig. 19. Extended body of *C. quissetensis*, showing digestive, gland and excretory systems, and reproductive system cell masses. × 200.

Fig. 20. *Cercariæum lintoni;* dorsal view showing digestive and gland systems, cell masses of reproductive system, and excretory vesicle. × 398.

Fig. 21. Sporocyst of *C. lintoni*, containing germ balls and mature cercariæ. × 70.

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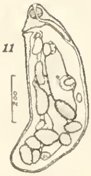

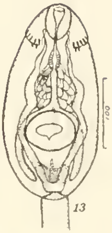

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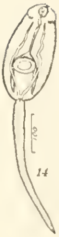

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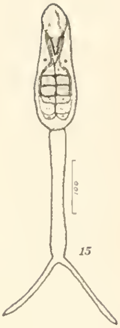

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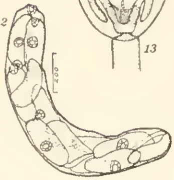

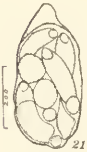

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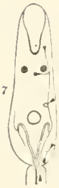

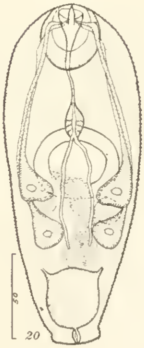

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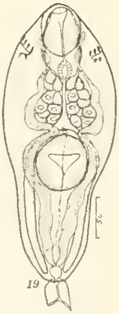

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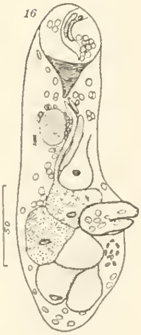

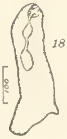

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CORRELATIONS AND VARIABILITY OF THE CENTRAL NERVOUS SYSTEM AND BODY SIZE OF THE ALBINO RAT.

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A knowledge of inter-organ weight relationships gives data which assist in the making possible a more exact conception of differential development. The presence or absence of a statistical association may indicate the presence or absence of a community of growth response to general developmental factors; it may indicate the presence or absence of a specific conditioning of the one organ by the other; or it may indicate the play of reciprocal influence.

The use of the coefficient of correlation in an investigation of this problem is marked by its neglect. What little the literature does contain, is, with one or two exceptions, sketchy and inadequate. It is usually a mere record of figures which in many cases are of meagre value because of the paucity of raw data.

Realizing the need for a more systematic survey of inter-organ weight relations a beginning was made somewhat over a year ago with a biometrical analysis of the weight interrelations of the glands of internal secretion. A preliminary note was published in Endocrinology (1) in anticipation of full presentation in the *Journal of Metabolic Research* at some future date. In the latter there will be found the conditions which should govern a study of this nature. The present paper is an extension of the analysis to the brain and spinal cord, using as raw data the body weights, body lengths, brain weights and spinal cord weights of the same rats the organ weights of which served for the earlier study. These animals were the controls of the studies of the thyroid apparatus (2). They were 150 days of age at the time the organs were removed for weighing. Both sexes were used, 121 rats of each. They all came from the Experimental Colony Stock of The Wistar Institute. The values thus represent the associations found in the mature male and female albino rat of this stock.

Turning now to the method of analysis I can do no better than to quote directly from Miner (3): "A coefficient of correlation measures the degree to which two variables are associated, taking the value + 1 when a deviation of one variable from its mean is always associated with a proportional deviation of the other in the same direction, decreasing as the intensity of the association decreases until for complete independence of the two variables it takes the value 0, and again increasing in numerical value, but with a negative sign for increasing intensity of association where deviations of one variable in one direction are coupled with deviations of the other variable in the opposite direction. Thus the absolute magnitude of a coefficient of correlation measures the intensity of the association of two variables, while its sign indicates whether as one variable changes the average values of the other variable change in the same or opposite directions. The possible range of values is from + 1 to − 1."

The coefficient of correlation does not of itself indicate which is the dependent and which the independent variable. This must be determined by other means. The simple or zero order correlation between any pair of variables is not representative of their association uncomplicated by assumed interferences derived from the other variables. In order to obtain such an uncomplicated index it is necessary to determine the correlation between any given pair of variables when the others are held constant. This is done by the method of partial correlation.

Now it is conceivable that the weight association between brain and spinal cord is factored by the general size factors carried by the body weight and possibly by the body length, particularly in the case of the spinal cord. Hence the elimination of these general factors for size by stabilization for body weight and length, is essential if we are to obtain the uncomplicated association between the brain and the spinal cord. That is to say the association free from the general body size factors. This has been done as succeeding paragraphs will show.

In order that the values obtained by the method of partial correlation be valid it is necessary that all the zero order regressions be linear, and that the number of observations in each of the zero order tables be fairly large as compared with the number of variables dealt with. The test for linearity is made through a determination of the correlation ratio and a comparison of this with the coefficient of correlation r. The methods used for the determination of the coefficient of correlation, the correlation ratio and the partial correlation coefficient are those described by Pearl (4).

The raw data and the correlation tables which serve as the basis of this study are not given because of space limitations. They are on file at The Wistar Institute.

In Table I. are given the zero order coefficients of correlation (r_0), the correlation ratios (η), the values for ($\eta^2 - r^2$), the probable errors for r_0 and ζ, and the quotients r/E_r and ζ/E_ζ of the several comparisons made in this study. In addition to these values there is to be found in the literature the following figures: Donaldson (5), (6) in a series of rats of scattered ages and with the sexes combined found the correlation between body weight and brain weight to be 0.764: the correlation between body weight and cord weight to be 0.856: between body length and brain weight to be 0.86: between body length and cord weight to be 0.99. Hatai (7) records a value of 0.516 in the male and of 0.692 in the female for the coefficient of correlation between body weight and cranial capacity in the mature albino rat. Jackson's (8) values are based on too few observations to be any more than suggestive. For adult man, Boas (9) records a correlation between body weight and head length of 0.43 in the male and 0.41 in the female; and between body weight and head width of 0.32 in the male and 0.33 in the female. Blakeman (10) found the coefficient of correlation between body length and brain weight to be 0.289 in the male and 0.367 in the female; while Pearl (11) got values ranging from 0.166 to 0.183 in the male and from 0.183 to 0.349 in the female for this association, and 0.167 in the male and 0.226 in the female for the degree of association between body weight and brain weight.

The values for man are of little help in the problem because the raw data from which they were derived are complicated by a multiplicity of interfering variables which vitiate any comparison with the figures obtained from the more uniform population of albino rats.

TABLE I.

THE COEFFICIENTS OF CORRELATION, THE CORRELATION RATIOS AND THE QUOTIENTS OF THE ERRORS OF THE SEVERAL COMPARISONS.

Subscript.	Male.					Female.				
	r_0.	r/E_r.	η.	ζ.	ζ/E_ζ.	r_0.	r/E_r.	η.	ζ.	ζ/E_ζ.
12	0.872 ± 0.015	58.1	0.882	0.0175 ± 0.0161	1.1	0.783 ± 0.024	32.6	0.811	0.0446 ± 0.0255	1.8
13	0.676 ± 0.033	20.5	0.725	0.0686 ± 0.0310	2.2	0.710 ± 0.030	23.7	0.755	0.0659 ± 0.0305	2.2
14	0.809 ± 0.021	38.5	0.821	0.0195 ± 0.0170	1.2	0.726 ± 0.029	25.0	0.770	0.0658 ± 0.0305	2.2
23	0.650 ± 0.035	18.6	0.701	0.0689 ± 0.0310	2.2	0.571 ± 0.041	13.9	0.632	0.0734 ± 0.0316	2.3
24	0.778 ± 0.024	32.4	0.798	0.0315 ± 0.0215	1.5	0.719 ± 0.030	24.0	0.751	0.0470 ± 0.0260	1.8
34	0.800 ± 0.022	36.4	0.813	0.0210 ± 0.0176	1.2	0.779 ± 0.024	32.5	0.799	0.0316 ± 0.0215	1.5

(1) Body Weight; (2) Body Length; (3) Brain Weight; (4) Spinal Cord Weight.

Notwithstanding the non-elimination of the age factor, the associations reported by Donaldson (6) between body weight and brain weight, body weight and spinal cord weight, and the brain and spinal cord in his series of rats are of the same order of magnitude as those found in this study. The association between cranial capacity and body weight in the female as recorded by Hatai (7) is also of the same order of magnitude as my value for body weight and brain weight correlation. Aside from these similarities the data are rather widely divergent.

Turning now to the analysis of my data it is evident that there is a high degree of positive correlation between the several pairs of variables under investigation. Neglecting the brain-spinal cord weight correlation ($r_{3\,4}$) it is seen that the order of increasing degree of association is the same in both sexes. This indicates that the association of the brain and spinal cord with the body as a whole is governed by factors which are largely independent of sex determinants of association. These independent factors are probably specific in origin.

The degree of association between brain weight and spinal cord weight ($r_{3\,4}$) is practically the same in the female as in the male. This indicates that the correlation is independent of the sex differences in body size which exist in animals of the same age. That is to say the association between these two parts of the central nervous system is independent of sex factors contributive to differences in differential development. It is, however, dependent on other factors. While it is possible that specificity plays an important part in the determination of the association, I am inclined to believe that the basis of the reaction lies rather in the community of characteristic chemical make-up of the two organs, with the consequent similarity in response or resistance to extraneous influences. Not to be neglected is the idea that the chemical similarity conditions a similarity in the processes of growth and hence association in weight.

The association between body weight and body length ($r_{1\,2}$) is consistently greater than that between the other pairs of variables. Nevertheless, the superiority is statistically valid in but 50 per cent. of the comparisons. The general trend of difference is uniform, however, and if accepted as significant is suggestive of a greater interdependence or dependence on a common factor. Body weight and body length are thus more closely related in differential development, than is the brain or spinal cord related to either of them individually. That is to say, body weight and body length follow more nearly parallel courses during differential development than do brain and body weight or body length, and spinal cord and body weight or body length. The difference can be attributed to the probability that the type metabolism productive of body weight is more nearly like that productive of body length, than is the type metabolism of body weight and body length like that which characterizes brain and spinal cord. In the case of body weight and body length the reactions are essentially increments in protoplasm, in the case of the brain and spinal cord the differential development is characterized by lipoid accretions.

The correlation between brain weight and body weight ($r_{1\,3}$) is less than that between the other pairs of variables with the exception of brain weight and body length ($r_{2\,3}$). The degree of difference is valid in 50 per cent. of the cases. The difference if accepted as significant is suggestive of a lesser dependence of brain weight and body weight on a common factor. That is to say brain weight and body weight are less related in differential development than are spinal cord weight and body weight and length, and than brain weight and spinal cord weight with each other. From which it can be inferred that during differential development brain weight deviates more from the course followed by body weight, than it does from that followed by the spinal cord, or than does the spinal cord deviate from the course followed by body weight and body length.

The greater association between brain and spinal cord ($r_{3\,4}$) is undoubtedly due to the community of type metabolism as compared with the disparity between brain and body weight. The higher degree of association between spinal cord and body length ($r_{2\,4}$) is justly attributed to the structural relations existing in this case. Why cord weight should be more closely associated with body weight ($r_{2\,4}$) than is brain weight is a question. It may be that the preference is a consequence of the structural relationship of the cord with the body length of high degree of correlation with body weight.

Both brain and spinal cord are more closely associated with body weight than with body length. Although the differences are slight and not statistically valid, the consistency of their direction and their presence in both sexes puts into the relation a significance that cannot be denied. It is a relation that might be expected by virtue of the fact that the developmental processes productive of weight in the parts of the organism are more closely allied than the developmental processes productive of weight are allied to those of length. This result would seem on the face of it to detract somewhat from Donaldson's (5) dictum that "body length is a better datum than body weight from which to infer the weight of the brain or spinal cord." The objection is negatived, however, by the fact that in a normal population the variability in body weight is greater than that in body length. This will be discussed presently.

The high degree of positive correlation of brain weight and spinal cord weight with body weight and body length allows the extension of Donaldson's (6) conclusion that the weight of the spinal cord can be inferred from body length or body weight with a high degree of accuracy, to include the brain.

With one exception (r_{13}) the degree of association between the several pairs of variables is greater in the male than in the female rat. The degree of difference is, however, statistically valid only in the case of the body weight-body length correlation (r_{12}). These figures in Table I., it must be remembered, are indices of the degree of association between pairs of variables, when interfering influences assumed to be exerted by the other variables are still present. Such being the case, and if the general trend of sex difference is accepted as significant because of its consistency, it is suggestive of a greater independence of the central nervous system of the female from the general factors contributive to interstructural and inter-organ association as carried by the body as a whole. This inference is supported by the results of the analysis by the method of partial correlations. The use of this method is allowable here because the regressions are in all cases essentially linear.

In Table II. are given the correlation coefficients of the several pairs of variables after the removal of the assumed influences exerted by the others by statistical treatment. Continuing the phase of comparison dealing with the sex differences. If we take as a measure of this relation the sex difference in degree of change in association of the first and second order coefficients from the zero order correlation between brain weight and spinal cord weight ($r_{3\,4}$), *i.e.*, when first body weight ($r_{34\,1}$) or body length ($r_{34\,2}$) are held constant, and then when the body weight and body length are both held constant ($r_{34\,12}$), it is seen that in general the reduction in value is percentagely greater in the male than in the female. This indicates that the conclusion drawn in the preceding paragraph is justified.

TABLE II.

THE (PARTIAL) CORRELATION COEFFICIENTS.

Subscript.	Male.	Female.
r_{12}	0.872	0.783
r_{13}	0.676	0.710
r_{14}	0.809	0.726
r_{23}	0.650	0.571
r_{24}	0.778	0.719
r_{34}	0.800	0.779
$r_{12\,3}$	0.773	0.654
$r_{12\,4}$	0.659	0.546
$r_{13\,2}$	0.293	0.515
$r_{13\,4}$	0.082	0.334
$r_{14\,2}$	0.425	0.377
$r_{14\,3}$	0.606	0.391
$r_{23\,1}$	0.169	0.034
$r_{23\,4}$	0.074	0.025
$r_{24\,1}$	0.253	0.353
$r_{24\,3}$	0.566	0.532
$r_{34\,1}$	0.584	0.545
$r_{34\,2}$	0.616	0.644
$r_{12\,34}$	0.655	0.573
$r_{13\,24}$	0.043	0.384
$r_{14\,23}$	0.325	0.069
$r_{23\,14}$	0.027	0.201
$r_{24\,13}$	0.193	0.399
$r_{34\,12}$	0.567	0.570

(1) Body Weight; (2) Body Length; (3) Brain Weight; (4) Spinal Cord Weight.

Further confirmation of the conception is had from the fact that the growth of the brain and spinal cord of the female albino rat follows the changes in growth retardation in body weight and body length which are caused by thyroid and parathyroid removal at different ages, to a lesser degree than does that of the male.

A general analysis of the progress of partial correlation would naturally only be an extension and confirmation of the comparisons made from the zero order values. As an example: the association between brain weight and spinal cord weight is conditioned to a lesser degree by body length than by body weight. This is shown by the fact that the percentage reduction in degree of association between brain and spinal cord weight from the zero order value ($r_{3\ 4}$), is greater when body weight is held constant ($r_{34\ 1}$) than when body length is held constant ($r_{34\ 2}$). The difference is what is to be expected from the fact noted earlier that brain weight and spinal cord weight are more closely associated with body weight than with body length.

It is important to note that there is a high degree of positive correlation between brain weight and spinal cord weight ($r_{34\ 12}$) which is independent of the general factors for size carried by the body as a whole. Indeed this value is much higher than that found for any of the pairs of organs so far studied (thyroid, adrenals, hypophysis, gonads, thymus and pancreas (1)). Between many of these no correlation was present at all after stabilization for body weight. Such being the case it is evident that the uncomplicated weight association between brain and spinal cord is peculiar. The phenomenon can be attributed to the community of characteristic chemical (lipoid) make-up of the two organs as already noted. As far as simple correlation with body weight is concerned, the brain and spinal cord have also a higher degree of association than any of the above, save the hypophysis and the pancreas in the male. The value of r_0 is 0.701 ± 0.031 for the hypophysis, and 0.600 ± 0.039 for the pancreas. It might be noted that since the same animals served as original sources of the material used in both studies, the value of the comparisons is enhanced.

There is no sex difference in the degree of association between brain weight and spinal cord weight freed from the general factors for size ($r_{34\ 12}$). The significance of this relation has been discussed in an earlier paragraph.

Another statistical value of assistance in an estimation of the forces concerned in differential development is the coefficient of variability when used comparatively. This figure is the quotient times 100 of the mean of the variates into their standard deviation, or, C.V. = $\sigma/M \times 100$ per cent. It is an abstract value which makes possible inter-group, inter-sex, inter-structural and inter-organ comparisons of sensitivity to the totale of forces contributive to variation which play upon the organism. In a study such as this, where a comparison is being made of the organs as parts of a whole, the differences in the coefficient of variability exhibited are indices of differences allied to differential development. They are worthy of investigation because they represent deep-seated biological relationships.

In Table III. are given the coefficients of variability and their probable errors of the body weight, body length, brain weight and spinal cord weight of the male and female albino rats at 150 days of age.

Table III.

Coefficients of Variability of the Body Weight, Body Length, Brain Weight and Spinal Cord Weight of the Male and Female Albino Rats 150 Days of Age.

Structure.	Male.	Female.
Body Weight	14.19 ± 0.63	11.39 ± 0.49
Body Length	4.42 ± 0.19	3.40 ± 0.15
Brain Weight	5.19 ± 0.22	4.92 ± 0.21
Spinal Cord Weight	6.69 ± 0.29	6.25 ± 0.27

The values show definitely that body weight in the female is less variable than in the male. This difference has already been noted by King for albino stock (12), for inbred albino stock (13) and for Norway rats (14). Jackson's (8) values show a like direction of difference in the albino rat.

This sex difference is not exhibited in adult man (11). Nevertheless it must be remembered that our data are derived from a racially homogeneous stock, while that of Pearl were not so constituted. It would be rash to state that the results of biometrical analysis of groups of humans is inadequate to divulge such relations. It is better to say that the lack of sex difference in value in a heterogeneous stock is no indication of its nonexistence in an homogeneous population. While it would also be rash to generalize from rat to man, the fact that three observers of five different rat populations have obtained sex-differences in body weight variability in the same direction, is indication that we are dealing with a biological sex-difference of response to factors contributive to variability, and that studies on man should be so planned as to eliminate the possible interfering factor of racial heterogeneity which might well mask a basic sex difference. Further evidence supporting this view is the fact that the body weight variability of the rat of homogeneous stock is generally considerably less than that of man of heterogeneous stock. The values recorded by Pearl (11) for man are 21.3 for the male and 24.7 for the female.

The female albino rat is also less variable than the male in body length to a statistically valid degree, and shows a like tendency in brain and spinal cord weight, though the degree of difference here is too small to be valid. At this time I do not want to go into a comparison of the entire array of organs in the rat, but might point out that the sex difference is not uniform in direction for all the organs, which fact has interesting implications as a later study will show.

However, the fact that in body weight and in body length the female is less variable than the male albino rat indicates in this species, at least, a greater stability of the female organism as a whole to outside forces tending to disturb body size equilibrium. Teleologically this might be considered an expression of a protective mechanism, tending to enhance resistance and thus favor the essential purpose of the female, namely reproduction.

From Table III. it is seen that body length is much more stable than body weight. The same holds true for man. The C.V. for stature in the male was found to be from 3.8 to 4.3 (Pearl) and 3.6 to 4.5 (Blakeman): and in the female from 4.0 to 4.7 (Pearl) and 3.8 to 4.2 (Blakeman). Attention is directed to the fact that the values for man are of the same order of magnitude as those for the rat.

It is hardly necessary to point out that the lesser variability of body length or stature is a consequence of the greater inherent metabolic stability of the skeleton as the chief component determinative of this measurement, as compared with that of the body weight with its predominant element of metabolically variable tissue. The one is, by virtue of its chemical make-up, a relatively fixed structure, the other a fluctuating mass freely subject to many influences.

The fact that the body length variability of the rat is of the same order of magnitude as that of man is indicative of a biological similarity in inherent structural response to factors contributive to variability, which might well have been predicted from the very nature of the structures involved, and which is support for the idea expressed in a preceding paragraph that the species difference in body weight variability exhibited here is factored in part by difference in racial uniformity of population from which the data were derived.

The lesser variability of the body length combined with the fact that the variability in brain weight and spinal cord weight is closer in degree to that of body length than to that of body weight establishes the conclusion postulated by Donaldson (5) that body length is a better datum from which to infer brain and spinal cord weight, than is body weight.

Both brain and spinal cord are less variable than the body in weight. This also holds for man in the case of the brain. Pearl (11) records a C.V. value of 7.5 to 8.8 for the male brain, and 7.1 to 8.7 for the female, while Blakeman (10) found 7.8 for the male and 8.2 for the female.

The lower variability of the central nervous system is obviously again an expression of an inherently more stable chemical make-up. Evidence for this is had both in the fact that the brain and spinal cord are more resistant to conditions of malnutrition and inanition than is the body as a whole (15), and in the fact that the growth of these organs is more resistant to the metabolic upsets incident to thyroid and parathyroid deficiencies than is that of the body in weight (16). This has been discussed in another paper. All that need be pointed out here is that the high content of the central nervous system in characteristically stable lipoids determines in it a resistance to metabolic disturbances which primarily affect the more readily utilizable tissue components, such as occurs in inanition, thyroid deficiency and individual dietary idiosyncrasies affecting body weight.

As one after another point of view is used in the attack on the problem of differential development, there emerges a unanimity of inter-relationship which substantiates the premises on which the interpretations are based.

The variability in brain weight is greater in man than in the rat. This is probably another expression of the difference between heterogeneous and homogeneous material. It is, therefore, not essentially a real difference. Contributive to, it may be the greater variability in human body weight as compared with rat body weight, and the high correlation between brain weight and body weight in the rat. The low association between this pair of variables reported for man rather negatives this idea, however.

Both brain and spinal cord are more variable than body length. In man also brain weight is more variable than stature. This may be taken as an indication that skeletal composition is more fixed and metabolically stable than is that of the central nervous system. Such a conclusion is in accord with the chemical and physiological data so far available.

The brain appears to be less variable than the spinal cord. This is consistent with the fact that brain weight is less highly correlated with body weight of high variability than is the spinal cord. On the other hand when body length variability is eliminated from brain and spinal cord variability free from the assumed influence of body weight variability, during the computation of the actual or reduced variability of these organs according to the usual statistical procedure, there is no reduction in value (of brain and spinal cord variability) below that which obtains when the variability has been stabilized for body weight. A different result would be expected if the respective brain and spinal cord associations with body weight and body length were dominant factors in the variability coefficients. Moreover, since the spinal cord has a relatively higher percentage of the stable (non-readily utilizable) lipoids characteristic of the central nervous system, and a relatively lower percentage of the labile readily utilizable elements than the brain, it would be expected that its (the spinal cord) variability would be less than that of the brain, if this compositional difference is the factor of importance. All the evidence so far accumulated gives an affirmative answer to this last point (17), and hence the reversal of expected relationship is either a real objection to the theory or else some other factor has intruded to mask the normal reaction. Such a factor is present in the technic of removal of the spinal cord for weighing. This is no place to describe the matter, nor is it necessary, for a little consideration will show that when the removal of an organ is accompanied by the cutting of many connections, the technic (no matter how much care is exercised) is bound to give a higher weight variability, than when the removal is merely a matter of shelling an organ out of its envelope. In view of this fact the brain-spinal cord difference in variability gives no basis for an inter-organ biological interpretation.

In a preceding paragraph mention was made of "reduced variability." This is a statistical value purporting to show the variability of a variable freed from influences assumed to be exerted by the other variables being studied. I have calculated these values for the body weight, body length, brain weight and spinal cord weight, and have arranged them in the order of decreasing variability in Table IV. as a matter of record. While in every case the variability is less than that which obtains when all factors of influence are in play, the relative position of any variable in the general scheme is unchanged.

Table IV.

The Reduced Variability of the Several Variables Discussed in this Study.

Structure.	Male.	Female.
Body Weight	11.41	9.86
Spinal Cord	5.28	5.06
Brain	4.35	4.08
Body Length	3.65	2.93

In the earlier study (1) on the weight inter-relationships of the glands of internal secretion a similar computation was made of the reduced variability of that particular array. It was found that the thyroid has the highest variability of all the organs so far studied, and that the thymus comes next. Both of these organs are much more variable than the body as a whole. The adrenals, gonads, hypophysis, and pancreas give lower figures, but in all cases values considerably higher than those for brain or spinal cord.

This generally lesser variability of the central nervous system is a demonstration that it is much more metabolically stable than are the other organs of the body so far studied. The interpretation is based on the difference in chemical make-up already discussed, in which differences in type metabolism participate. A more extended discussion of the problem is reserved for a later paper.

Summary and Conclusions.

The brain and spinal cord of the albino rat show a high degree of positive weight correlation, free from the influences assumed to be exerted by the other variables studied (body weight, body length). The value for the male is 0.567, and for the female 0.570.

The weight variability of the brain and spinal cord is less than that of any of the other organs so far studied. It is greater than that of the body in length.

The analysis and interpretation of these and other relations are given in detail in the text.

The computations upon which this study is based were made by Miss Mildred Wilson.

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THE NUTRITION OF THE OVUM OF *HYDRA VIRIDIS.*

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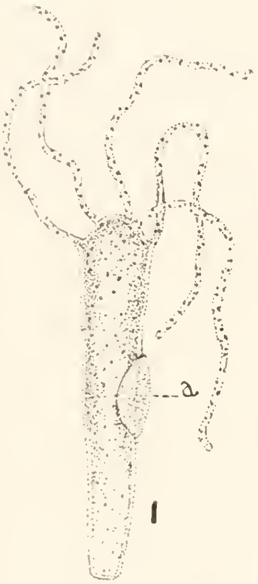
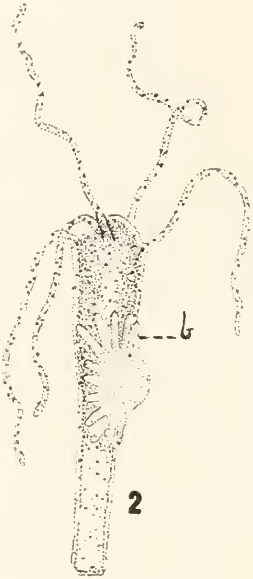
The incipient ovary of *Hydra viridis* is represented by a mass of proliferated interstitial cells. At a very early stage in the development of the ovary some of its cells become much larger than the others. There seems to be a struggle on between these cells; for, as development of the ovary proceeds, but one of the larger ones remains whole, and the smaller ones perish. The cells, that are directly involved in this struggle, are enclosed in a thin wall of slightly chromatic, modified epithelio-muscular cells. Kleinenberg (72), Brauer (91), Downing (08) and Tannreuther (08) agree in the statement that one of the enlarged interstitial cells gets the ascendency over the others and grows at their expense. If there be in the incipient ovary more than one greatly enlarged interstitial cell, these may fuse to form the oögonium as over against the cells of what Tannreuther (08) designates "the cells of the peripheral region which contribute to the formation of the yolk," p. 274. These peripheral cells are not taken into the growing oögonium's cytoplasm bodily as Brauer (91) described. They disintegrate at the periphery of the oögonium and are then resorbed. The relation of these disintegrating cells to the cytoplasm of the final oögonium are shown in Fig. 1. Figs. 3 and 4 show phases of disintegration in these neighboring enlarged interstitial cells. As their cytoplasm breaks down, the nuclei display disintegration features. Eventually the entire cell disintegrates. The substance of these disintegrated cells is absorbed by the oögonium, as Kleinenberg (72) described. The material thus obtained results in the oögonium growing greatly to become a conspicuously large amœboid cell (Text-figure 2). This cell, by means of radiating stout pseudopods, spreads out over one third or more of the mesoglea's outer surface. This amœboid gamete was first figured by Fewkes and Mark in 1884. This pseudopodial cell has now attained its maximum size and is, therefore, the primary oöcyte. The feeding of the oögonium, of the final oögonial generation, upon the neighboring, enlarged interstitial cells represents the first phase of the nutrition of the ovum of *Hydra viridis.* Nutrition, in this phase, is referred to the growth of the final oögonium into the primary oöcyte.





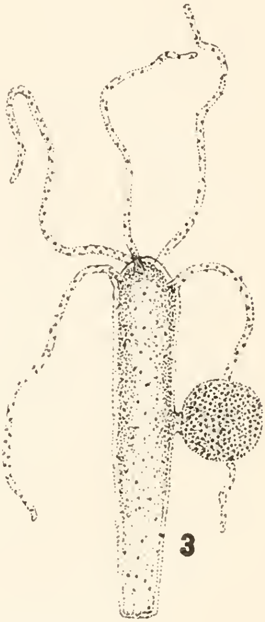


FIG. 1. Oögonium before pseudopodia are thrown off. *a* shows plane through which section, shown in figure 1 of plate, was taken.

FIG. 2. Oöcyte with maximum number of pseudopodia. *b* shows plane through which section, shown in figure 5 of plate, was taken.

FIG. 3. Advanced primary oöcyte with a full complement of deutoplasm.

Previous investigators have failed to recognize that there are two phases in the nutrition of *Hydra's* ovum. Perhaps failure, on their part, to recognize the dual nature of the nutrition of the ovum of *Hydra* arises out of the resemblance of the disintegrating interstitial nuclei to deutoplasmic granules (Fig. 3). Brauer (91) held that the nuclei of the cells, that were being ingested by the egg, became the yolk granules or his "Pseudozellen." Downing (08) says the "interstitial cells adjacent to the egg in the fairly mature ovary have their walls in contact with the egg resorbed and the content of the cell becomes part of the egg (Nusbaum).

The greatly enlarged nuclei, gorged with lecithin, also become yolk granules or "Pseudozellen," p. 66. Tannreuther (08) also looks upon the yolk granules as arising from the nuclei of the interstitial cells. He says: "When the pseudopodia are completely formed, the nuclei of the interstitial cells forming the ovary are taken up by the amœboid egg and become changed into the yolk or pseudo-cells of the egg. . . . After the yolk or pseudo-cells are formed they divide amitotically" (p. 264).

We have been unable to bring the details of *Hydra viridis* ovary in line with the above interpretations and facts.

To begin with, up until the time that the growing, amœboid oögonium has made maximum contact with the mesoglea—thus placing it in relation to the endoderm—there has been no yolk formation. In the meantime, however, many of the attending, peripherally disposed cells have disintegrated and have been resorbed. Sometimes this disintegration and resorption has gone so far that, before the oögonium has well advanced in growth, the attending cells lie only at its margin (Fig. 1). In a typical example, by the time the oögonium has reached its maximum growth, there is but a thin tissue of highly modified epidermis covering its general surface (Fig. 5). In such example, there may yet be disintegrating cells at the tips of the pseudopods or even beyond them. Thus it is to be emphasized that many of the enlarged interstitial cells have disintegrated (perhaps most of them) and have been resorbed during the oögonium's growth. And yet, up until maximum surface contact with the mesoglea has been established, no yolk formation has resulted. It should be further indicated that we have found no enlarged interstitial cells to be taken up bodily by the amœboid egg-cell. These two features of the nutrition of ovum of *Hydra* stand in contrast with what Tannreuther (08) describes. He says: "When the egg has reached its growth, it is amœboid in form with the nucleus near the center. The egg at this stage of development contains no yolk, . . . but when pseudopodia are completely formed, the nuclei of the interstitial cells forming the ovary, are taken up by the amœboid egg and become changed into yolk or pseudo-cells of the egg. Fig. 6 represents a cross-section of several pseudopodia into which the nuclei of the interstitial cells of the ovary are passing" (p. 263–264). The contrast between what Tannreuther herein describes and our observations appears in two ways. In the first place, if yolk formation depends upon the disappearance of the interstitial cells of the ovary, then yolk should appear when these cells disappear. They are clearly seen to disappear throughout the growth of the oögonium and yet until the latter has reached its full growth no yolk has appeared. In the second place, we find that no interstitial cells have been bodily taken up or ingested as shown by Tannreuther in his Fig. 6. His Fig. 6, however, is not, in itself, convincing; for he shows the so-called nuclei leaving only two interstitial cells. Moreover, the cells from which these nuclei are migrating show no marked cytoplasmic change. Likewise, his written observations are not convincing with reference to the manner in which yolk arises. He makes the significant statement that "The pseudopodia do not grow out between the cells of the ovary, but rather between the ovary as a whole and the mesoglea" (p. 263). If, now, the pseudopodia were sent out with reference to yolk formation, dependent upon the interstitial cells of the ovary, they would "grow out between the cells of the ovary" and not "between the ovary as a whole and the mesoglea."

There is no meaning in the extensive application of the primary oöcyte's pseudopodia to the mesoglea, if the yolk granules are derived from the interstitial cells. On the other hand, we see in this spreading out of the primary oöcyte over the mesoglea a method of making maximum contact with a source of food material upon which to draw for the elaboration of deutoplasm.

In *Hydra*, the endoderm is the source of food supply. Kepner and Hopkins (24) observed that, as a diploblastic animal, *Hydra* cannot transport widely material absorbed by the endoderm. For example, chloretone injected into the enteric cavity of *Hydra* effects only the adjacent ectoderm of the body proper. The sphincters at the bases of the tentacles prevented the injected chloretone entering the latter and the compression of the walls of the peristome prevented chloretone entering its lumen. It was thus of interest to observe that the tentacles and peristome received none of the chloretone that had been absorbed by the general endoderm, for they became unusually active in contrast to the quieted body proper. Just as chloretone could not be sent to the closed tentacles, so it appears the endoderm of *Hydra* cannot send food-material along a narrow channel to its oögonium. The growing oögonium must, therefore, come to the endoderm. As a result of this imposition, by the time the final oögonium has become, through growth, a primary oöcyte, an extended relation between the latter and the endoderm has been established. This relation established marks the inception of the second phase of the nutrition of the ovum of *Hydra viridis*. At the beginning of this second nutritional phase, there are no deutoplasmic inclusions within the cytoplasm. Soon, however, yolk is formed within the cytoplasm of the oöcyte (Fig. 5). This deutoplasm is elaborated by the oöcyte out of material taken over in solution from the endoderm and assimilated by the female gamete. Thus the deutoplasm may be looked upon as material elaborated by the oöcyte. The deutoplasmic granules are not to be considered the lineal descendants of original nuclei of neighboring interstitial cells that have come to be more and more numerous through amitosis. This position seems logical when we bear in mind the fact that, though many interstitial cells have disintegrated (perhaps most of them) and have been resorbed during the egg's growth, yet, up until maximum surface exposure to the endoderm has been made, no yolk-formation has resulted. Our interpretation is further strengthened by the observation that so long as yolk is making its appearance within the primary oöcyte a maximum surface relation to the endoderm is maintained; but when the maximum amount of yolk has been formed the egg retreats from the endoderm as Tannreuther (08) indicates: "After the amœboid egg becomes filled with yolk, the pseudopodia are drawn in and the egg becomes nearly spherical" (p. 264), (Text-figure, 3). The second phase of the nutrition of *Hydra viridis*, therefore, ends with the retreat of the primary oöcyte from the mesoglea after it has become filled with deutoplasm. No deutoplasm is formed thereafter. This phase of nutrition is referred to the development of the zygote.

SUMMARY.

The nutrition of *Hydra viridis* is a dual process, there being two phases.

The first phase has reference to the nutrition of an oögonium of the final generation. This oögonium is nourished through the disintegration and resorption of adjacent interstitial cells. Through the nourishment, thus obtained, the final oögonium grows into a large pseudopodial cell, the primary oöcyte. The first nutritional phase is referred to the growth of the final oögonium into a primary oöcyte. It does not involve yolk-formation.

The second phase of nutrition begins with the primary oöcyte lying, as a pseudopodial cell, in extended relation to the endoderm. Yolk is elaborated by the oöcyte from material handed over by the endoderm and the protoplasm of interstitial cells is not involved. The second nutritional phase is referred to the development of the zygote.

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EXPLANATION OF PLATE.

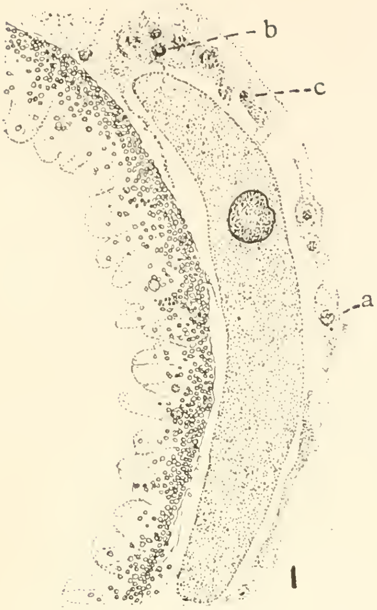
FIG. 1. Part of a section taken through plane indicated at *a* in text-figure. A group of disintegrating interstitial cells is shown at *b*, *c*. *a* shows that already, in this particular example, the superimposed ectoderm was but a single layer thick. × 250.

FIG. 2. Cell *a* of Fig. 1, magnified to indicate the character of cell that forms ovarian wall. × 1,250.

FIG. 3. Cell *b* in Fig. 1. Shows a disintegrating interstitial cell in which the nucleus resembles a deutoplasmic granule. × 1,250.

FIG. 4. Cell *c* in Fig. 1. An attending interstitial cell in a more advanced phase of disintegration than cell shown in Fig. 3. The nucleus no longer resembles a deutoplasmic granule. × 1,250.

FIG. 5. Section taken through plane indicated at *b* in text-figure. Shows eleven pseudopodia closely applied to mesoglea. Yolk-formation has begun; it is not, however, completed though all enlarged interstitial cells have disappeared. × 250.



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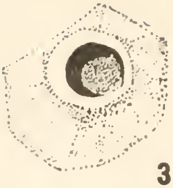

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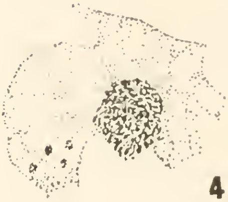

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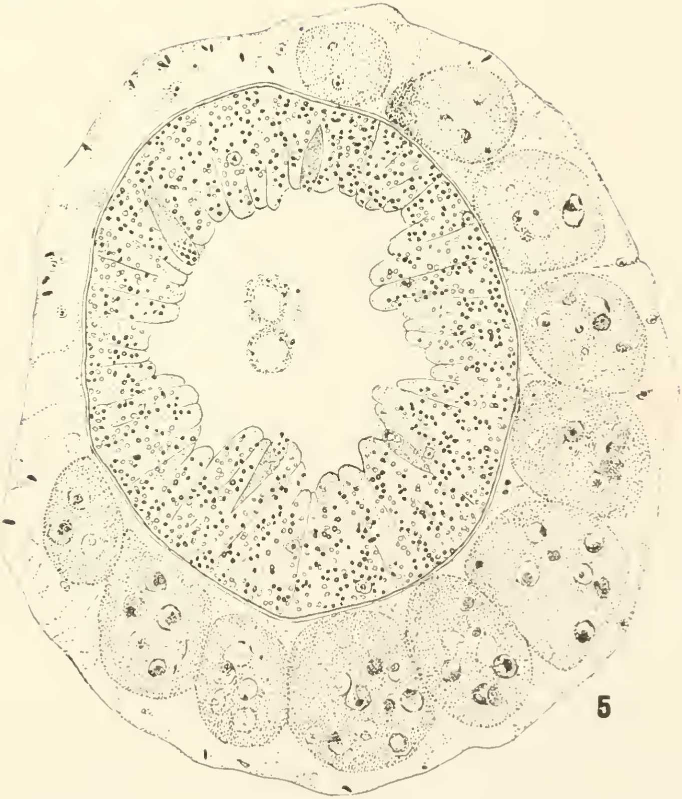

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